Genetics of Australian lizard fish

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Statements

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ABSTRACT

The population and evolutionary genetics of fishes of the genus *Saurida* from waters off northern Australia were examined. Allozyme electrophoresis was used to examine the population structuring of two species. The phylogenetic relationships of five species were investigated by sequencing sections of the mitochondrial genome.

General proteins and 18 enzymes representing 21 gene loci were initially screened to examine resolution of banding patterns and the levels of polymorphism. Taxa could be separated on the basis of banding patterns for general proteins and *LDH**.

Twelve loci were used to examine population structuring in samples of *Saurida* sp. 2 collected from the North West Shelf, the Gulf of Carpentaria and off the east of the Cape York Peninsula. Contingency chi-square analyses of homogeneity of allele frequencies at eight loci indicate significant stock structuring among the three areas sampled. This structuring was supported by gene diversity analysis.

Saurida undosqamis samples were examined from North West Shelf and off the east of the Cape York Peninsula; the species is absent from the Gulf of Carpentaria. Based upon frequencies of the *MDH** locus, there were significant differences between two temporally separate samples collected from the North West Self. Contingency chi-square analyses of homogeneity of allele frequencies at the five variable loci showed a genetic stock structure among the three samples (two North West Shelf and one Cape York). Significant differentiation was detected at *GPI-2**, and *MDH**.

For phylogenetic analyses, the mitochondrial cytochrome oxidase *c* subunit 1 gene (CO1), cytochrome *b* gene and 12S RNA gene were sequenced from the five groups of saurids. Parsimony analysis confirmed that *Saurida* sp. 2 is a sister taxon to S. *undosquamis*. and that *S.* cf *argentea* is more closely related to *S. filamentosa.* The relationships of *S. longimanus* remained unresolved.

CHAPTER 1 INTRODUCTION

1.1. Lizardfish

1.1a. Synodontidae

The Synodontidae (lizardfish) is a conspicuous element of the demersal fish fauna of tropical and subtropical seas. Until recently, the family name Synodontidae has been used for two distinct groups of fish: lizardfish in the Order Myctophyformes and upside-down catfishes in the Order Siluriformes. Clarification of the use of Synodontidae was made by Russell (1987) who clearly demonstrated that it must apply to the myctophiform lizardfish by chronological priority. He evoked articles 23 (chronological priority), 29 (formation of family group names) and 60 (replacement of junior homonyms) of the International Code of Zoological Nomenclature to determine that the siluriform upside-down catfish family should be given the name Mochokidae.

The number of genera in the family Synodontidae remains unsettled due to complex relationships among the members of the family. Norman (1935) recognized five genera —*Synodus, Saurida*, the monotypic *Trachinochepalus*, *Bathysaurus* and *Harpodon*. Others (e.g., Marshall , 1964 reviewed by Adjei, 1984) excluded *Bathysaurus* and *Harpodon* from the family, while Sulak (1977 reviewed by Adjei, 1984) added the genus *Xystodus*. Johnson *et al.* (1996) removed *Saurida* and *Harpodon* and placed them under the family Harpadontidae due to the close relationship between *Saurida* and *Harpadon*. They also rejected *Bathysaurus* and preserved *Synodus* and *Trachinochepalus* in the family Synodontidae.

Despite this controversy, the three genera *Synodus, Saurida, Trachinochepalus* are widely recognized in the Family Synodontidae (Last, pers. comm.). The family consists of about 50 species and is found in tropical and subtropical regions (Golani, 1993), in both the Atlantic and Indo-Pacific region from Japan and the Philippines through the Indian Ocean to the Red Sea and eastern Mediterranean.

Lizardfish generally inhabit sandy and muddy bottoms. However, some have also been found on shallow coral reefs and others on deep reef areas where sandy channels or pockets adjoin rock and coral ledges. Most lizardfish bury themselves in sand while a few typically hide in rock crevices or under ledges. Some species occur in estuaries.

Most Synodontidae species occur at depths of 1 - 200 m, but a few species do inhabit deeper water. Variation in depth distribution between species plays a major role in maintaining niche separation in lizardfish. Golani (1993), in a study of the biology of *Saurida undosquamis* in the eastern Mediterranean Sea, showed that although it and *Synodus saurus*, were both piscivores and equally abundant, the former species occurred in deeper water than the later. Another case was a study of the two stocks of *Saurida undosquamis* in Japanese waters. The south stock was found at a depth of about 200 m at the edge of the continental shelf while the north stock generally inhabited muddy bottoms in depths of less than 150 m (Yamaoka *et al.*, 1989).

Lizardfish have a reptile-like head with prominent jaws. The mouth is large with numerous slender sharp teeth and inward directed teeth on the tongue. This appearance testifies to their activities as voracious piscivores (Thresher, 1984; Randall *et al.*, 1990). They feed on a wide variety of small and sometimes not so small fish, both ambushing and chasing prey (Golani, 1993). The feeding behaviour of lizardfish may significantly affect mortality rates of sympatric fish species. The small bodied lizardfish, *Synodus englemani,* has been reported from Lizard Island, Great Barrier Reef, to attack (although not always succesfully) other fish on average once every 35 min and consume 650 fish per year (Sweatman, 1984). Salini *et al.* (1994), in a study on diets of trawled predatory fish in the Gulf of Carpentaria, observed that *Saurida* sp2, *S. micropectoralis* and *S. filamentosa* did not consume commercially important penaeid prawns, although the former consumed small and non-commercial species of prawns.

The spawning seasons of lizardfish species are not well investigated (Thresher, 1984). Golani (1993) reported that *Saurida undosquamis* breeds all year in the Gulf of Oman, but a population of the same species in the eastern Mediterranean Sea established some time this century via migration from the Gulf of Oman through the Red Sea and Suez Canal, breeds only from March to December. This curtailment of the breeding period is assumed to be a result of the lower water temperatures in the new habitat. Synodontids were reported to have pelagic eggs that range from small to medium size (0.9-1.5 mm) and spherical with hexagonal reticulations on the chorion (Leis and Rennis, 1983). However, *Saurida undosquamis* has been found to have smaller ripe oocytes (0.5-0.6 mm) (Golani, 1993).

Planktonic synodontid larvae are extremely elongated and round to ovoid in cross-section. They have distinctive pigment patches on the long and straight gut which has rugae, they lack a conspicuous gas bladder and have high myomere counts. The variation of black pigment spots along the gut permits their identification at species level. Prior to settlement this pigment becomes less distinct as it becomes internal and further pigment may occur on the brain and body. Settlement to the bottom occurs when the larvae are 30 - 35 mm long and takes place at night (Leis and Rennis, 1983; Thresher, 1984)

The genus *Saurida* Cuvier and Valenciennes, 1849, is morphologically very distinct from the other two genera (*Synodus*, *Trachinochepalus*). Distinctive features are: the palatine teeth arrangements with a double band of teeth on each side of the palate; inner rays of pelvic fins not much longer than outer ones; and pelvic bones with short, laminar posterior processes (Norman, 1935; Shindo and Yamada, 1972). *Synodus* and *Trachinochepalus* are more closely related (Johnsone *et al.*1996) but they have some distinguishing characters that separate them. In *Synodus* these are: snout as long as, or longer than eye; anal fin with 8 to 15 rays compared with 15 to 16, its origin much nearer to the base of the caudal fin than to head; and vent nearer to base of caudal fin than to insertion of pelvic fin (Norman, 1935).

The current taxonomy of *Saurida* is based largely on the works of Norman (1935), Matsubara and Iwai (1951), Shindo and Yamada (1972) and Waples (1981).
At the moment, there are 11 recognized Indo-Pacific species of the genus *Saurida* (Adjei, 1984). The species are:

1. *S. argentea* Macleay, 1882
2. *S. australis* Castelnau, 1878-9
3. *S. elongata* Temminck and Schlegel, 1846
4. *S. filamentosa* Ogilby,1910
5. *S. flamma* Waples, 1982
6. *S. gracilis* Quoy and Gaimard, 1924
7. *S. isarankurai* Shindo and Yamada, 1972
8. *S. longimanus* Norman, 1939
9. *S. nebulosa* Valencienes, 1849
10. *S. tumbil* Bloch, 1795
11. *S. undosquamis* Richardson, 1848

However, there are problems in determining the number of valid species in the genus

Saurida. Previously, other Indo-Pacific species of *Saurida* were recognized but they were not included by Adjei (1984) in the list above. These were:

1. *S. micropectoralis* Shindo and Yamada, 1972
2. *S. wanieso* Shindo and Yamada, 1972

Some described species are separated only on relatively minor morphological differences and there are probably some nominal species which contain two or more cryptic taxa. The existence of a two or more species "*undosquamis*" complex and the confusing relationship between *S. elongata* and *S. tumbil* have long been known (Last, pers. comm.).

Some possible distinct species in the "*undosquamis*" complex were recorded (Norman, 1935). These included:

1. *Saurida undosquamis* Richardson, 1848
2. *S. grandisquamis* Günther, 1864
3. *S. argyrophanes* Günther, 1864

Such uncertainties may complicate the study of species especially when they occur in sympatry. Therefore, the accurate delineation of species boundaries is an important precursor to any population studies.

Lizardfish occur in northern Australian waters. Sainsbury *et al.* (1985) recognized 5 species in the genus *Saurida* and 7 species in the genus *Synodus* in waters on the continental shelf of northern and north-western Australia. The genus *Saurida* was represented by *Saurida* sp1, *Saurida* sp2, *S. longimanus, S. undosquamis* and *S. micropectoralis*. The genus *Synodus* was represented by *S. indicus, S. jaculum, S. hoshinonis, S. macrops, S. variegatus, S. saganeus, S. kaianus*. The monotypic *Trachinochepalus*, *T. myops* also occurred in these areas. Salini (1994) recognized three species of *Saurida* in the Gulf of Carpentaria, i.e. *S. micropectoralis, S. filamentosa,* and *Saurida* sp2.

1.1b. Commercial fisheries

Lizardfish from the genus *Saurida* include several economically important demersal species in a number of countries including Japan, Thailand, India and Israel. The three most economically important species are *S. undosquamis* (the most commonly caught species), *S. tumbil* and *S. elongata*. The fish are used both for stock feed and human

consumption. In Japan, the fish are used as a raw material for fish paste. In Israel, the fish are used for food and poultry feed. In Australia, the fish are not economically important and are not consumed, but they are a significant component of the by-catch from prawn and fish trawling. Also, foreign vessels from Japan, Taiwan and Thailand have extensively exploited the fish from the groundfish resources of Australia's northern trawl fishery (Ramm and Xiao, 1994). So they are important as a potential resource for an export-oriented fishery.

Ramm and Xiao (1994) reported that lizardfish was one of the major catch components on the Australian North West Shelf with a total annual catch of 4276t in 1973. In a study of the demersal fish fauna from the Australian North West Shelf, Thresher *et al.* (1986) showed an increased number of *Saurida* spp in the catch during a 5 year period (1978-1983). The increase in *Saurida* sp2 numbers was spectacular with the population size nearly doubling every year. This was far greater than the increased number of *Saurida undosquamis* and *Saurida* sp1. The changes to population sizes of *Saurida* may be due to commercial trawl fishing that reduced the number of competitors and/or predators, or increased the area of open sand habitat favored by lizardfish.

The occurrence of *Saurida* spp in the demersal trawl fisheries was also reported from the Gulf of Carpentaria. The Gulf was closed to all fish trawling in 1979 to prevent any posssible damage to the valuable prawn-trawling grounds but reopened for domestic fish trawling in 1991. Blaber *et al.* (1994) reported that the overall mean catch rates of fish were 421.3 kg h^{-1} for day trawls and 198.6 kg h^{-1} for night trawls. Based on mean percentage of catches, the absolute mean catch rates of *Saurida* sp2 were 9.9 kg h^{-1} and 10.6 kg h^{-1} for day and night trawls, respectively.

1.1c. Studies on saurids

The identification of species within the lizardfish genus *Saurida* has been, and continues to be in a state of confusion. This is mainly due to the fact that most studies to date have relied upon external and internal morphological features only and many taxa have proven difficult to distinguish. The application of molecular techniques has proven to be very valuable but, as yet, has been used only sparingly in studies of the *Saurida* genus.

Norman (1935) reviewed the lizardfish of the world and recognized nine species of the

genus *Saurida* from the warmer parts of the Atlantic and Indo-Pacific Ocean. Later, Matsubara and Iwai (1951) examined three of the more commercially important species, *S. undosquamis, S. tumbil, S. elongata.* Some inconsistencies were found in the studies of these fish. Matsubara and Iwai (1951) reported some incongruences with the previous studies on *S. tumbil.* The outer band of the palatine teeth of the fish was always set in 2 rows instead of 3 rows as it had been observed by Norman (1935). On the basis of this feature and a filamentous second dorsal ray in the adult male, Matsubara and Iwai (1951) proposed that *S. filamentosa* was synonymous with *S.tumbil.* However, this observation was rejected by Shindo and Yamada (1972) who retained *S. filamentosa* as a valid species.

Waples (1981), in the study of lizardfish genus *Saurida* in Hawaiian waters, proposed that *S. nebulosa* should be removed from the synonymy with *S. gracilis.* One character that distinguish both species was the palatine teeth arrangements. In *S. nebulosa* the inner series of palatine teeth consisted of two quite distinct rows while in *S. gracilis* it consisted of three poorly defined rows. Other characters that can be used for separating the two species include pigmentation on gill filaments and peritoneal, pectoral fin, and upper jaw. Allozyme electrophoretic data showed that fixed allelic differences were found between *S. nebulosa* and *S. gracilis* at 10 presumptive gene loci.

New species have subsequently been added to Norman's (1935) list of *Saurida.* Shindo and Yamada (1972), in a morphological study of the lizardfish genus *Saurida*, reported three new species. These were *S. wanieso* that was caught from the East China Sea, *S. isarankurai* and *S. micropectoralis* from Gulf of Thailand. Waples (1981) reported a new species (*S. flamma*) in Hawaiian waters. This identification was based on both electrophoretic and morphological data.

Several stock differentiation studies of *Saurida* species have been carried out using morphometric characters. For example, Avsar *et al.* (1987) applied the Mahalanobis distance function for the morphometric separation of *S. undosquamis* stocks in the Gulf of Mersin. This study was based on eleven morphometric characters and suggested one unit stock existed in this region.

Some studies of interspecific and intergeneric comparison of lizardfish have been

carried out using protein electrophoresis. Taniguchi (1969) found that *S. undosquamis* differs markedly from *S. elongata,* and that *S. tumbil* is intermediate between these two species. Shaklee *et al.* (1982) reported that the genetic distance between genera (*Synodus* versus *Saurida*) was not significantly larger than that between the most divergent *Synodus* species. The Nei's distance values at the nodes connecting taxa were spread rather evenly over the range 0-2.0.

In a study of life history strategies, Thresher *et al.*(1986) showed that there were two distinct size classes of female *S. undosquamis* on the North West Shelf of Australia. The larger body size was identified as *S. undosquamis* while the smaller body size was reported as *Saurida* sp2. The two species were different in their patterns of growth and sexual maturity — *S. undosquamis* growing to a moderate size over several years while *Saurida* sp2 reached sexual maturity at a younger age and may rarely live more than a year.

While the above situation was about two morphologically similar species of *S. undosquamis* from Australian waters, a similar situation also involving *S. undosquamis* was recognized in the East China Sea. Early studies in this region (Yamada and Ikemoto, 1979) showed that there were two types (south and north) of *S. undosquamis* that were different in their morphology and ecology. The specific status of both types was not clear until allozyme electrophoretic analysis was carried out by Yamaoka *et al.* (1989). In their study, the two types of *S. undosquamis* were confirmed to be distinct species with fixed allelic differences at eight loci. Nei's genetic distance between the two types was 0.5582 which is within a range of differentiation at specific level. However, no specific name was given to the second species.

There is a lack of suitable data on lizardfish in Australian waters that make management of the species difficult. These problems can be categorized into three groups — species identification, phylogeny and population structure.

1.2. Species identification

Species identification may be carried out by morphological or molecular approaches. Most of the data on species identifications are usually provided by morphological approaches. For example, Creech (1992) used multivariate morphometrics to investigate

whether *Atherina boyeri* and *A. presbyter* were morphometrically distinct. The results were consistent with the expectation of the existence of two species. From the within group covariances, females of the two species were different in a large number of morphological characters. Males were different in a more restricted number of characters such as head 'shape' and body 'shape'. Serventi *et al.* (1996), in the study of mullet fry (Mugilidae) from Italian coastal waters, showed that pharyngobranchial morphology was useful to identify larger fry or juveniles. For the smaller fry, the use of pigmentation characters proved effective. Urho (1996), in the study of Perch (*Perca Fluviatilis*), Pikeperch (*Stizostedion Lucioperca*) and Ruffe (*Gymnocephalus Cernuus*) larvae, demonstrated that myomer counts from yolk to anus could distinguish the three species at the early yolk sac stage. Another useful feature was the characteristic line-shaped melanophore pattern between the myomeres. In the later larvae, other characters that could be used for identification were the position of the mouth, the length of the jaws, the number of fin rays in the anal and second dorsal fin. Anderson and Heemstra (1980) used counts and measurements to demonstrate two new species of Western Atlantic *Anthias.*

Morphological approach, however, is often inadequate, especially in the case of closely related species that are morphologically similar, or when morphological characters have been removed once the fish was landed. In addition, when unknown tissue samples, larvae, or suspected hybrids are examined, molecular approaches become the choice to address these problems.

Isozyme electrophoresis has been the predominant tool to address problems of species identification in fisheries. For example, the cryptic species of arrow squid (*Nototodarus*) from New Zealand waters were thought to be a single species, however, allozyme electrophoresis showed that there were two species characterized by fixed differences at three loci (Smith *et al.*, 1987). A similar result was reported in the genus *Photololigo* from northern Australian waters (Yeatman and Benzie, 1994). Also, morphologically similar fish species have been identified as separate species, e.g. the blue maomao *Scorpis violaceus* and sweep *Scorpis aequipinnis* in New Zealand waters (Smith *et al.*, 1979), and *Atherina boyeri* and *A. presbyter* in Europe (Creech, 1991). In Taiwanese waters, Lee *et al.* (1997) demonstrated that morphologically similar elvers of *Anguilla japonica* and *A. marmorata* can be distinguished easily, with fixed allelic differences

found at *CK-D** and *LDH-B**.

Moreover, isozyme electrophoresis has been used to resolve the two body forms of fish that were thought to be a single species such as sprat *Sprattus antipodum* (Smith and Robertson, 1981). Another problem that has been addressed with isozyme electrophoresis is the identification of larvae, such as those of snapper *Chrysophrys auratus* (Smith and Crossland, 1977), and rockfish from the genus *Sebastes* (Seeb and Kendall, 1991).

Sometimes, combining both morphology and isozyme electrophoresis was useful, for example, Kelsch and Hendricks (1986), in a comparative study of the American catfishes *Ictalurus*, demonstrated that multivariate morphometric analysis and isozyme electrophoretic data support the separation of *I. lupus* and *I. punctatus*. Another example is the work of Creech (1991, 1992) on the genus *Atherina*.

Although the use of morphological and isozyme electrophoresis techniques were proved useful in the identification of many species, sometimes the techniques failed to resolve the problems. In such cases, DNA-based species identification methods such as restriction mapping or sequence analysis of mitochondrial DNA (mtDNA), restriction fragment length polymorphism (RFLP), Polymerase Chain Reaction (PCR) and random amplified polymorphic DNA (RAPD), have proved useful.

A number of studies have been carried out to identify fish using DNA-based identification methods. Asahida *et al.* (1997) in a study of the stomach contents of sand shrimp, *Crangon affinis*, reported that PCR and restriction analysis of mtDNA were successful in distinguishing larvae and juveniles of the stone flounder *Kareius bicoloratus* from other prey. Bartlett and Davidson (1991) reported that PCR and direct sequence analysis of the mitochondrial cytochrome *b* genes have proved useful for separating four species of tuna in the genus *Thunnus*. Partis and Wells (1996) used RAPD methods to identify fillets of eight species of fish (barramundi, Nile perch, john dory, mirror dory, silver dory, spikey oreo, warty oreo and smooth oreo).

In other studies, DNA-based identification methods have had limited success. For example, Chow *et al.* (1993) in a study of western Atlantic snappers , used PCR-RFLP

analysis of mitochondrial cytochrome *b* and 12S ribosomal RNA to identify thirteen species in the subfamily Lutjaninae. The result showed that eight species could be identified by haplotype analysis on either single or both fragments while other species were not separated from one another because of overlapping or identical haplotypes.

1.3. Phylogenetic reconstruction

Phylogenetics is a study of the evolutionary history of species by reconstructing the topology of the tree of life. This is carried out by grouping sister species based on joint possessions of uniquely derived homologous characters called synapomorphies. This procedure may result in single or monophyletic groups since all organisms share certain hereditary features (Bremer and Wanntorp, 1979; Avise, 1994).

Morphological methods have been used to determine the evolutionary relationships in many species. For example, Eastman (1997) showed that osteology was useful in determining the evolutionary relationship in the Antarctic nototheniid fish *Pleurogamma*. Baldwin *et al.* (1997) demonstrated that the lineage of flashlight fish *Protoblepharon* could be explained with some morphological characters, such as number of gill rakers on the first arch, body-scales rows, and the occurrence of postorbital papillae. Didier *et al.* (1998) used the early morphological development to describe the three major chondrichthyan lineages (sharks, batoids, and chimaeras).

Despite the great contribution of morphological studies in systematics, morphological characters are often not sufficient for phylogenetic studies. For example, the stable inheritance of characters is required, and phylogenetic information which is not influenced by environmental factors is best suited. These characters are generally obtained from genomic information such as nucleic acids (Swofford and Olsen, 1990).

Basically, there are two kinds of genetic data for inferring phylogenetic relationships i.e., discrete characters and similarities or distances (Swofford and Olsen, 1990). Discrete characters include allozyme electromorph mobilities, restriction endonuclease fragment lengths, DNA sequences and RAPD markers. These data will give information about individual species. Two kinds of terminology are recognized in discrete character data, the character and its state. A character is an independent variable that consists of a number of character states. States maybe binary with a presence or absence, e.g.

restriction site (recognized by restriction endonuclease) or a particular allele at an isozyme locus, or they may be multistate, e.g. in DNA sequencing, where each nucleotide position represents four character states. There are several advantages with the use of character/state data, such as identifying homoplasious steps and recognizing actual branch lengths (Buth, 1984). Similarity or distance data provide information about quantitative pairwise relationships between taxa or molecules. This data may be derived from immunological cross-reactivities and DNA hybridization profiles.

Sometimes it is preferable to use similarity or distance methods rather than character-based methods. Character data can be analyzed using distance methods with an appropriate transformation (Swofford and Olsen, 1990). However, there may be a resulting loss of information. Transformation of distance data to character data is not possible. So, data from immunological cross-reactivities and DNA hybridization profiles can only be analysed using distance-based methods.

There are four common methods for the estimation of evolutionary trees, i.e. distance-matrix, parsimony, invariants and maximum likelihood (Swofford and Olsen, 1990). The most widely used method to infer phylogenies from character data is based on the principle of maximum parsimony.

Isozyme electrophoresis has been used to study the biochemical systematics of some fish. For example, Sole-Cava *et al.* (1983) showed that there was a clear genetic difference between two sibling species of *Squatina* in South Brazil. Lacson and Bassler (1992) studied fish of the genus *Stegastes* from the southern Marianas using 14 presumptive loci (10 of which were polymorphic). The results accorded with the morphological partition of *Stegastes* into at least two subgenera. Stephien (1992) identified the synapomorphies of the family Clinidae in the Eastern Pacific by allozyme analysis. The results suggested that synapomorphies of matritrophy separated the subfamily Clininae from the Tribe Myxodini. Keenan (1991) used 40 loci to study the phylogeny in 24 taxa of Australian flatheads (Platycephalidae), and Elliott and Ward (1995) studied the genetic relationships of eight species of Pacific tuna. Lowry *et al.* (1996) inferred phylogenetic relationships of seven Oreosomatidae species from Australian waters using allozyme analysis.

Combining both morphological and isozyme electrophoresis approaches has been done

in some species. For example, Miller *et al.*(1994) used both methods to show that the Italian freshwater gobies do not form a monophyletic group.

Direct sequencing of mitochondrial DNA following PCR amplification provides a powerful tool in phylogenetics studies, in particular, the mitochondrial cytochrome *b* gene is commonly used. For example, Orti *et al.* (1994) showed that there were two major divergent clades in the threespine stickleback *Gasterosteus aculeatus.* One clade was widespread in Japan (but also representative in Alaskan and British Columbian lakes) and the other in Europe and North America. Lockwood *et al.* (1993) inferred phylogenetic relationships among members of the Coregoninae. The results supported the previous classification of Coregoninae and suggested that the genera within this subfamily radiated 2-6 million yr B.P. Schmidt *et al.* (1998) studied the phylogenetics and evolution of the cytochrome *b* gene in the North American cyprinid genus *Lythrurus.*

Miya and Nishida (1996) studied phylogenetic relationships of the deep-sea fish genus *Cyclothone* using mitochondrial 12S and 16S ribosomal RNA sequence analysis, and described in detail the evolutionary history of the genus. Ritchie *et al.* (1997) investigated the phylogenetics of Antarctic notothenoid fishes with 12S and 16S mitochondrial DNA sequences, and 28S, 12S and 16S rDNA. The results showed that the family Bovichtidae was paraphyletic and *Pseudaphritis* is the sister group of all the non-bovichtid notothenioids. Murphy and Collier (1997) carried out phylogeny studies of aplocheiloid fish using cytochrome *b,* 12S rRNA, and 16S rRNA. The results accorded with the monophyly of the Neotropical family Rivulidae and positioned the basal sister group of Indo-Malaysian and Madagascaran taxa relative to a monophyletic South American/African dichotomy. This suggested the role of vicariance in the diversification of the fish. Waters and White (1997) used mitochondrial sequence analysis to clarify phylogenetic relationships between Tasmanian mudfish, *Galaxias cleaveri,* and the morphologically similar New Zealand mudfish genus, *Neochanna.* The results supported the current morphological allocation of the Tasmanian mudfish to *Neochanna.* Ovenden *et al.* (1997) inferred the phylogeny of red and green rock lobster genus *Jasus* using 16S RNA and cytochrome oxidase subunit 1 gene. Cladistic analysis of nucleotide substitutions showed that monophyly of species within each of the *lalandii* and *frontalis* subgroups of Jasus was not supported.

1.4. Population genetics

There are two basic types of population structures — demographic and genetic (Slatkin, 1994). The demographic structure of a population can be influenced by processes such as birth, death, dispersal, mating system and life history. Such data can be obtained from censusing and observation without knowledge of the genetic basis of any of these characters. The genetic structure of a population can be influenced by isolation and mutation processes. An understanding of the pattern of genetic variation in the species is important to determine the genetic population structure. Data can be obtained by assessing the genetic makeup of different individuals within the population using a choice of molecular markers.

The study of population genetics was greatly influenced by the development of electrophoresis techniques. Initially, protein variation was used to estimate the level of genetic variation within a species and to describe population structure. This approach remains a powerful tool to study population structure in most taxa despite recent advances with mitochondrial DNA and nuclear DNA markers (Parker, 1998). There are three kinds of measure of genetic structure of populations, i.e. allelic, genotypic and gene pool measures (Rothe, 1994). These measures are used to describe the pattern of genetic exchange among subpopulations and the degree of isolation among local geographic units.

The information about the genetic structure of a fish population or stock can be used by fisheries managers to regulate fishing to ensure sustainability of the fishery, preserve depleted stocks and conserve the genetic variability of the species.

1.5. Morphological markers

Morphological characters are widely used in population and systematics studies. A huge amount of information exists from morphological studies using meristic and morphometric measurements, e.g. sharks and rays (Compagno, 1977), Atlantic *Anthias* (Anderson and Heemstra, 1980), American catfishes, *Ictalurus* (Kelsch and Hendricks, 1986).

Morphological approaches have been used to study population structure in some fish. Haddon and Willis (1995) used morphometric and meristic comparison to study orange

roughy, *Hoplostethus atlanticus*, in New Zealand waters. The result showed significant morphological differences between samples from the Puysegur Bank and Lord Howe Rise. The same species was studied by Elliott *et al.* (1995) on the continental slope of Australia. Their results suggested that there were at least seven morphologically distinguishable stocks. Henault and Fortin (1989) compared spring and fall spawning ecotypes of cisco, *Coregonus artedii*, in southern Quebec, Canada. Morphometric and meristic analysis indicated that genotypic differences occurred between these stocks. Yano and Musick (1992), in a morphometric study of Atlantic and Pacific specimens of false catshark, *Pseudotriakis microdon,* found little difference between oceans.

There are several advantages with the use of morphological markers over genetic markers for taxonomic studies. Historically, the greatest advantages were the ability to use the extensive collections of preserved specimens in museums, and the applicability to fossil species (Avise, 1974) since there is a low percentage of biomolecules preserved. However, modern DNA techniques make it possible to study DNA from preserved and fossil materials (Yang, 1997). Moreover, morphological studies can use the advantage of ontogenetic information to distinguish phylogenetically informative data from phylogenetic noise in systematics (Hillis, 1987). Ontogeny criteria are assumed to be more superior than outgroup criteria since the ontogenetic states can be observed directly and *a priori* assumptions of relationships need not be made (Nelson, 1978).

There are also some disadvantages in the use of morphological characters. One of the primary problems is nonheritable variation (Hillis, 1987) and convergence (unrelated organisms evolve to become superficially similar). Also, morphological data (body form) are often prone to subjectivity, and are often confounded by allometry or differences in body proportions because of the different sizes of the individuals (Waples, 1981).

Despite the increasing challenge from molecular methods applied to taxonomy, the output from morphological data has not reduced. This is mainly because each method is able to address questions and problems that cannot be addressed by other methods.

1. 6. Molecular markers

1.6a. Protein variation

Isozymes are a set of enzymes that have similar functions encoded by one or more loci. Variant forms of isozymes that are the products of alleles at the same locus are termed allozymes. Allozymes can be separated by electrophoresis techniques and the pattern of their mobility is a measure of their phenotypes. The electrophoretic variation has a simple genetic basis. The electromorph contains one or more polypeptides with a genetically determined amino acid replacement that changes the electrophoretic mobility of the enzyme (Hartl, 1991).

Isozymes have been used widely as molecular genetic markers and applied in a variety of organisms, such as plants (Testolin and Ferguson, 1997), fungi (Vogler and Bruns, 1998), insects (Karotam *et al.* 1995) and fish (Arculeo *et al.*, 1996). For evolutionary studies, differences in isozyme numbers and isoenzyme properties are used, while for quantitations of genetic variation among or within populations, allozyme frequencies are used (Rothe, 1994). Isozyme electrophoresis has been proved useful for defining genetic markers for stock identification in fish as evidenced by numerous studies that document differences in protein allele frequencies between stocks (Altukhov, 1981; Iles and Sinclair, 1982; Grant, 1985; Carvalho and Hauser, 1994).

Many studies of population genetics derived from isozyme electrophoresis resulted in the suggestion of a single genetically homogeneous population, e.g. Greenland halibut, *Reinhardtius hippoglossoides*, in the north-west Atlantic area (Fairbairn, 1981); Atlantic halibut, *Hippoglossus hippoglossus*, in Norwegian waters (Mork and Haug, 1983); Pacific halibut, *H. stenolepis*, in the Bering Sea and Gulf of Alaska (Grant *et al.*, 1984); Pacific Ocean perch, *Sebastes alutus*, in the Western Gulf of Alaska and the Bering Sea (Seeb and Gunderson, 1988); Spanish sardine, *Sardinella aurita,* from South Carolina to the Florida panhandle (Kinsey *et al.*, 1994); oil sardine, *S. longiceps*, in the western coast of India (Menezes, 1994); Atlantic herring, *Clupea harengus*, from the Northern Gulf of Bathnia to the North-east Atlantic off the west coast of Norway (Ryman *et al.* 1984) and from North American and European seas (Grant, 1984).

In Australian waters, a single large panmictic population was observed in some species through isozyme electrophoresis studies, e.g. blue grenadier, *Macruronus*

novaezelandiae, (Milton and Shaklee, 1987), orange roughy, *Hoplostethus atlanticus* (Elliott and Ward, 1992), jackass morwong, *Nemadactylus macropterus* (Richardson, 1982 ; Elliott and Ward, 1994), *Lutjanus sebae, Lethrinus nebulosus, Lethrinus choerorynchus,* and *Epinephelus multinotatus* (Johnson *et al.* 1993).

Isozyme electrophoresis has also revealed some disjunct populations, e.g. Pacific cod, *Gadus macrocephalus*, in the western and eastern North Pacific Ocean (Grant *et al.* 1987); Atlantic cod, *G. morhua*, in the Baltic Sea and the Atlantic Ocean (Mork *et al.*, 1985) and in the Baltic Sea and George Bank (Grant and Stahl, 1988); witch flounder, *Glyptocephalus cynogloccus*, in each of the major areas off Newfoundland (Fairbairn, 1981b); Pacific herring, *Clupea pallasi*, in Bering Sea and Gulf of Alaska (Grant and Utter, 1984).

Some genetic divergences were also observed in some species in Australian waters. For example, Mulley and Latter (1981) reported the geographic differentiation of tropical Australian penaeid prawn populations. Genetic differences was observed in *Penaeus latisulcatus* and *P. endeavori* from the Gulf of Carpentaria and Western Australia. Benzie *et al.* (1992), in the study of *Penaeus monodon* in Australian waters, showed that highly significant differences occurred between the west-coast population and those on the northern and eastern coastlines. Jerry (1997) reported that isolation by distance occurred in the catadromous Australian bass, *Macquaria novemaculeata*, based on 6 polymorphic allozyme loci. Shaklee *et al.* (1993) demonstrated that multiple genetic stocks of barramundi perch occurred in Queensland waters. Keenan (1994) reported that isolation by distance in a one dimensional stepping stone model occurred in the Australian barramundi (*Lates calcarifer*) population, and that genetic diversity decreased as the population spread into new habitat.

There are some advantages in the use of isozymes electrophoresis. The technique is easy, reasonably cheap and permits the relatively rapid collection of data (Ward and Grewe, 1994). Data can be collected from hundreds of individuals at several loci in a few days or weeks. One of the most important features of alleles coding for allozymes is codominance which means that heterozygotes express the allozyme corresponding to each allele (Buth, 1990). This allows an indirect assessment of the genotype from its biochemical phenotype. The benefits of the codominant expression of alleles include the

testing of the validity of presumed species or identifying distinct species. Other benefits include the identification of interspecific F_1 hybrids between two species having multiple fixed allelic differences, the measurement of genetic relatedness among individuals and the measurement of reproductive relationships in sympatric populations. Allozyme patterns are virtually unaffected by environmental variables such as temperature, salinity, and oxygen tension. Therefore, comparison can be made among specimens from different environmental backgrounds (Shaklee *et al.*, 1982). An additional benefit is derived from the molecular clock hypothesis (Carlson *et al.*, 1978; Nei 1971), which assumes that proteins evolve at relatively constant rates. The approximate time of divergence of any two species (or higher taxa) may be estimated with appropriate calibration using the values of genetic distance derived from electrophoretic studies.

However, there are some limitations in the use of isozyme electrophoresis. These include the number of loci resolved (Grant and Utter, 1980; Grant, 1984; Utter *et al.*, 1989) because of the limitation of histochemical staining techniques (Hunter and Markert, 1957; Morizot and Schmidt, 1990). Although more than 100 loci can now be visualized with histochemical staining (Wright *et al.*, 1983; Morizot and Siciliano, 1984), this is still only a very small sample of the total coded for by the genome (Lewontin, 1991). Also, there are restrictions emanating from the number of alleles per locus and number of individuals required for population or phylogenetic studies. The resolution of protein electrophoresis sometimes fails to detect differences between populations or individuals, and low levels of divergence may be observed in morphologically distinct species. Another limitation results from the redundancy in the DNA code that dictates protein sequences. Only a small percentage (±1%) of the whole genome in animals codes for essential proteins. Non-coding regions of the genome are often more variable since they are less subject to selective pressures. Also, high degrees of sequence homology may be found in coding regions of the gene between distantly related organisms (Park and Moran, 1994). In addition, not all changes in a gene coding for protein result in a change in the overall charge of the protein expressed. Consequently, many variations in the genome cannot be detected by isozyme electrophoresis.

Not withstanding the above, the ability of isozyme electrophoresis to derive acceptably

precise estimates of genetic parameters should ensure its continued widespread use and it is not obsolete, despite the advent of techniques which permit direct examination of the genome. Other molecular procedures need only be applied when isozyme electrophoresis cannot adequately resolve or identify differences among groups (Utter, 1994; Ward and Grewe, 1994). Moreover, the existing isozyme database can be used as a more practical source of genetic information at least until a substantial amount of DNA data has been collected for a particular species (Park and Moran, 1994).

1.6b. Mitochondrial DNA

In recent years, mitochondrial DNA (mtDNA) has become established as a powerful tool for population and evolutionary studies of animals and it is considered to be more powerful than isozyme analyses (Moritz *et al.*, 1987; Avise *et al.*, 1987; Ward and Grewe, 1994).

The popularity of the use of mitochondrial DNA comes from its unique properties. Mitochondrial DNA is easy to purify and isolate because of its unusual buoyant density, a high copy number and occurrence in an organelle other than the nucleus (Wilson *et al.*, 1985). Mitochondrial DNA is easy to characterize because of its small genome size (16 - 20 kb) compared to the millions of nucleotides in the nuclear genome. Also, it lacks complicated features such as repetitive DNA, transposable elements, pseudogenes, and introns (Avise *et al.*, 1987). Mitochondrial DNA is generally a duplex and a covalently closed circular molecule. A mitochondrial genome generally consists of 2 rRNA genes, 22 tRNA genes and 13 protein genes which code for subunits of enzymes functioning in electron transport of ATP synthesis. In addition, a displacement loop, D-loop or A+T rich region (about 0.8 kb long), exists that may have a function to control mtDNA replication and RNA transcription. Mitochondrial DNA is haploid and maternally inherited since almost no cytoplasm from the sperm is contributed to the fertilized egg (Barton and Jones, 1983). As a result of maternal inheritance, the patterns of distribution of mtDNA are more subdivided than nuclear DNA. The mode of genetic transmission in mtDNA is straightforward with no recombination. However, there was evidence that rapid genotypic shift might have occurred in the bovine mtDNA D-loop (Olivo *et al.* 1983). The pattern of mitochondrial inheritance in which alleles segregate during mitotic cell divisions may also result in evolutionary change (Birky, 1983). Although the mitochondrial gene content has been conserved for 350 my, some of its parts can evolve

rapidly with new character states commonly arising within the lifespan of a species. The rate of mutation is about 1 - 10 faster than a typical single copy of nuclear DNA (Brown, 1979; Wilson *et al.*, 1985; Vawter and Brown, 1986).

Despite the potential value of mtDNA analyses, some real limitations need to be recognized. One is the possibility, although rarely, of extensive heteroplasmy that may complicate mtDNA study. In theory, hundreds or thousands of mtDNA molecules exist in most somatic cells and mature oocytes. Heteroplasmic condition may result from a new mutation and two or more genotypes may coexist within an individual (Avise *et al.*, 1987). Another limitation results from homoplasy or similarity not directly attributable to common ancestry. Homoplasy or extra steps (reversal, parallelisms, convergences) may occur if a rapid rate of change occurs in a small number of interconvertible character states in which each nucleotide position is characterized by only four assumable states: adenine, guanine, thymine, and cytosine (Avise, 1994). Moreover, nucleotide positions in mtDNA evolve at a different rate from one to another position. A problem may occur when working only with the more slowly evolving portions of the molecule i.e. distinct populations may be misinterpreted as conspecific populations (Avise *et al.*, 1987).

Combining allozyme and mitochondrial DNA analyses has been used to study the population structure of fish in Australian waters. Ward *et al.* (1994) compared the Atlantic salmon cultured in Tasmania with their ancestral population in Canada. Both methods showed that the two samples were very similar. Elliott (1996) analysed the tropical saddle-tail sea perch, *Lutjanus malabaricus*. In this study, analysis data from 10 polymorphic loci and 20 composite mtDNA haplotypes suggested that multiple stocks (North West Shelf, Gulf of Carpentaria, east coast of Queensland) of *L. malabaricus* occurred across northern Australia.

Mitochondrial DNA variability alone has been used in some population studies in Australian waters. Ovenden *et al.* (1993)studied the Atlantic salmon, *Salmo salar*, brown trout, *S. trutta*, rainbow trout, *Oncorhynchus mykiss* and brook trout, *Salvelinus fontinalis* from Tasmania. The results showed that mtDNA variability was low. Smolenski *et al.* (1993) reported that there was evidence of stock separation in orange roughy, *Hoplostethus atlanticus* in New South Wales waters. In an investigation of jack

mackerel, *Trachurus declivis* from south-eastern Australian waters, Smolenski *et al.* (1994) observed that although the level of genetic diversity was low, there was evidence of temporal differences in the mtDNA diversities of two Tasmanian samples. Another species, jackass morwong, *Nemadactylus macropterus*, was studied by Grewe *et al.* (1994) and, as in a previous allozyme study (Elliott and Ward, 1994), no significant difference occurred among Australian samples but there was a significant different between these samples and New Zealand samples.

1.6c. Restriction fragment length polymorphisms (RFLPs)

RFLPs are used to detect polymorphism in DNA molecules directly. These markers are widely used to study natural populations, e.g. in the Nile tilapia, *Oreochromis niloticus* (Agnese *et al.* 1997), Pacific Salmon, *Oncorhynchus* spp (Moran *et al.* 1997), Atlantic cod, *Gadus morhua* (Fevolden and Pogson, 1997).

RFLP analysis may be approached in two ways, using either Southern blot hybridization or PCR (Parker *et al.* 1998). In the former method, a probe DNA that is complementary in sequence to a DNA segment of interest is needed. An RFLP results when genomic DNA is digested with a restriction enzyme. Fragments of different size are then separated by electrophoresis. The radioactive probe DNA hybridizes only with DNA fragments containing complementary sequences. As a result, the variation in these restriction fragments can be detected. However, the use of Southern blot hybridization to study RFLPs has some disadvantages, e.g. it is time-consuming and expensive. In the second approach, PCR primers are used to amplify random fragments of the genome. The PCR product is then treated with restriction enzymes to produce fragments that can be separated by electrophoresis. Although this approach is more feasible than the former approach, a great deal of labor is still required to construct a genomic DNA library, some initial sequencing, and a fine-tuning of the PCR condition (Parker *et al.* 1998).

1.6d. Random amplified polymorphic DNA (RAPDs)

RAPDs is a method used to create genomic fingerprints from species when its target sequence is not or only partially known. In this method, primers are randomly chosen from sequences of short oligonucleotides. Variation in genomic regions result from the presence or absence of complementary primer annealing sites (Parker *et al.* 1998). The RAPD patterns that arise can be used to determine the relationship among species or for

RFLP analysis. This is due to the degree of variability observed for many primers. Elo *et al.* (1997), in the study of nonanadromous salmon, *Salmo salar* and brown trout, *S. trutta*, demonstrated that the RAPD method was relatively quick and the least expensive method for detection of interspecific hybridization.

As with other markers, there are problems associated with RAPD markers. Landry and Lapointe (1996) reported that the method failed to compare taxa above family level. Also, RAPD markers lack codominant markers as in the case of RFLPs, and as a result, information on the parental origin of alleles may be unobtainable (Lewis and Snow, 1992). RAPD fragments are short and may produce some artificial amplification products, and careful control of DNA quality and amplification conditions is required (Scott *et al.* 1993).

1.6e. Nuclear DNA sequences

The nuclear genome provides a wealth of information, with 0.3-4.0 billion base pairs available for analysis (Park and Moran, 1994). At present, direct examination of nuclear DNA (nDNA) variability remains an undeveloped field. The reason for this is largely because of the difficulty that arises from recombination and heterozygosity that makes interpretation of data difficult. Also, the expense for developing suitable probes is high (Ward and Grewe, 1994).

There are many ways of classifying nDNA sequences, e.g. coding and non-coding DNA, repetitive and non-repetitive (Park and Moran, 1994). One source of nDNA markers that is commonly studied is satellite DNA. This repetitive and non-coding DNA contains tandemly repeated short nucleotide sequences or VNTR loci (variable number of tandem repeats). There are two classes of VNTR, i.e. minisatellites and microsatellites. The former refers to tandem arrays from ten to a hundred nucleotides long. These repeat sequences are highly polymorphic and are often GC rich (Jeffreys *et al.* 1985). Also, they have a high heterozygosity (close to 100%) and high mutation rates (more than 2% per generation) (Jeffreys *et al.* 1988). Microsatellites refer to smaller variable repeat sequences of one to four nucleotides (tens to hundreds of base pairs) (Wright and Benzten, 1994). Different kinds of repeats, such as a GT repeat and a GA repeat may compose microsatellites. Microsatellites also have high heterozygosities and high mutation rates (0.05% to 0.%) (Ward and Grewe, 1994).

Another repetitive and non-coding DNA sequence is the interspersed repeated DNA (Park and Moran 1994). LINEs and SINEs refer to long and short interspersed repetitive elements, respectively. Both sequences are not tandemly repeated and are smaller than satellite DNA. SINEs have been used in phylogenetic studies in some species, e.g. in Pacific salmonids (Murata *et al.* 1993), and in African chiclid fish (Takahashi *et al.* 1998). Bryden *et al.* (1998) reported that a SINE-like repetitive Element (ROn-1) is an abundant element in the genomes of many African cichlid fishes, but absent from the genome of the Indian cichlid *Etroplus.*

1.7. Molecular genetic techniques

1.7a. Gel electrophoresis

Gel electrophoresis is used very widely and extensively in protein and DNA research. The principle of the technique for both areas of research is the same. It is based on the separation of ionized or colloidal size molecules in an applied electric field. Both proteins and DNA can be separated based on differences in size and shape. Also, proteins can be separated based on the differences in the net surface charge due to the acidic and basic groups of some amino acids (Whitmore, 1990). This is not the case for DNA because DNA molecules are negatively charged. The separation of protein and DNA is usually affected by factors such as migration velocity, temperature and interaction between sample and buffer solution (Bruno, 1991).

The most commonly used electrophoretic technique is zone electrophoresis in which protein and DNA are distributed into discreet "zones" (Bruno, 1991). Previously, zone electrophoresis was run in a liquid solution. However, this practice is not popular since it has some disadvantages such as convection and diffusion in the zone (Hames, 1990). Currently, a medium stabilized within a supporting medium is used. Examples of media include high grade filter paper, cellulose acetate film, or gels made of starch, polyacrylamide, or agarose. Isozymes are usually run in cellulose acetate films and starch gels. DNA is commonly run in either agarose or polyacrylamide gels, depending on the size of the DNA fragments being separated (Park and Moran, 1994).

Other variations of electrophoretic techniques include continuous-buffer electrophoresis, discontinuous-buffer electrophoresis, isoelectric focusing, two dimensional techniques,

sodium dodecyl sulphate (SDS) and urea electrophoresis (Ferguson, 1980).

Isozymes and DNA can be visualized by applying several staining methods. A wide variety of staining methods is needed to visualize isozymes, as they depend on the specific locus being visualized and usually exploit the enzymatic action of the protein. DNA fragments are visualized using non-specific staining methods which means that they do not distinguish one sequence from another. Non-specific stains include dyes such as ethidium bromide, that when bound to DNA will fluoresce under ultraviolet light and produce a bright image against a dark background. Another dye for DNA staining is silver oxide, a reverse stain where a dark image will be visible in ambient light (Park and Moran, 1994).

1.7b. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a valuable technique that can be used to amplify a specific DNA sequence of interest (Mullis, 1990). The reaction is remarkably fast and can amplify a short stretch of DNA (usually fewer than 3000 bp) a million fold. The technique has been used for many applications including the direct sequencing of mitochondrial DNA (Bartlett and Davidson, 1991; Lockwood *et al.*, 1993) and examination of nucleotide sequence variation (Carr and Marshall, 1991; Miracle and Campton, 1995).

The PCR technique takes advantage of polymerase enzymes that catalyse the synthesis of new strands of DNA. Two conditions are generally needed for the polymerase enzymes to start synthesizing the new strands of DNA: firstly, a template of the desired DNA sequence, obtained by denaturing the two DNA chains of the double helix, and secondly, two oligonucleotide primers that flank the desired DNA sequence to be amplified. Each primer (short nucleotides of 10-30 bp) is complementary to the beginning of the sequence to be synthesized. The primer is needed as a starting point for the DNA polymerase to synthesize the new strands in a 5' to 3' direction. In addition to DNA polymerases, a supply of the four nucleotide bases is required. The four nucleotide bases serve as the building blocks of every piece of DNA (Erlich *et al.* 1988).

Basically, there are three steps in which PCR is performed. The first step is the denaturation (±94° C for 30 sec) of the double-stranded template DNA so that all the

DNA is single-stranded. The second step of the cycle (±55• C for 30 sec) involves the actual annealing of the primers to the template DNA. The annealing temperature is based on the nucleotide composition of the primer. As a rule of thumb, A's and T's are worth 2° C and C's and G's are worth 4• C. The third step of the cycle is the extension (±72• C for 30 sec) in which the polymerase starts to synthesize new strands (Palumbi *et al.*, 1990). The cycle can be repeated 30 or more times with each newly synthesized DNA piece acting as a new template. As a result, the number of DNA templates increases exponentially and 1 million copies can be ready in about three hours.

Initially, the Klenow fragment (PolI-Kf) of *Escherichia coli* DNA polymerase I was used to catalise the extension of the annealed primers. However, this proved to be tedious and an error-prone process if several samples were amplified simultaneously, as PolI-Kf is a thermolabile enzyme and needs to be added after each cycle. Therefore, a thermostable DNA polymerase that can survive at 95° C was necessary. Thermophilic bacterium, *Thermus aquaticus* (*Taq*) is a heat-resistant polymerase that is relatively unaffected by the denaturation step (Gelfand, 1989). *Taq* polymerase quickly replaced PolI-Kf as it simplifies the entire procedure. Other benefits of using *Taq* polymerase are that the specificity, yield, sensitivity, and length of targets that can be amplified are increased (Saiki *et al.*, 1988; Andersson and Gibbs, 1994).

1.7c. DNA sequencing

The direct determination of the sequence of bases in a DNA molecule may provide a wealth of information about gene structure and gene expression. This technique can be applied to different kinds of DNA markers such as single copy, satellite and interspersed repetitive sequence.

There are several techniques available for DNA sequencing. One of these techniques uses a chemical procedure to break a terminally labeled DNA molecule at each repetition of a base (Maxam and Gilbert, 1977). The different chemical agents would cleave the DNA molecule preferentially at guanines, adenines, cytosines and thymines equally. The position of the base can be identified by the lengths of the labelled fragments. The products of the four reactions were separated by polyacrylamide electrophoresis. The fragments were separated according to size and can be read from the pattern of radioactive bands. One of the advantages of the method is that the

chemical treatment is easy to control. The chemical attacks one base per strand producing a rather even distribution of labelled material across the sequence. Also, each base will be attacked and different bases will be clearly distinguished. One of the limitations of this method is the resolution attainable in the gel electrophoresis.

However, the most common method of DNA sequencing is the chain terminating inhibitor method (Sanger *et al.* 1977). In this method, a dideoxy (lacks the 3'-OH group) and arabinucleoside are used. The dideoxyribose acts as specific chain-terminating inhibitors of DNA polymerase by preventing the formation of the phosphate bond that will link the next nucleotide of the chain. Four kinds of dideoxyribose (ddATP, ddTTP, ddCTP, ddGTP) are required to terminate the chain when they are combined with adenine, thymine, cytosine, or guanine, respectively.

Four identical DNA synthesis reactions are performed in separate tubes. The reactions contain each of the four dideoxyribose in each tube. Other ingredients that are added in all four tubes include a single stranded DNA template of interest, a primer and all four deoxyribonucleoside triphosphates. Fragments that are produced by these reactions will have different sizes that correspond to the distance of nucleotides from the primer. These fragment need to be labelled either by radioactive or fluorescent detection methods so that they can be determined by gel electrophoresis. The base sequence can be read by the relative positions of the fragments.

There are several methods for the detection of the DNA fragments.The conventional method uses radioactivity that is inexpensive and generally successful on templates of poor quality, however, it is a time consuming method (Andersson and Gibbs, 1994). The latest method uses fluorescent automated DNA sequencing that is more accurate and more sensitive (Heller, 1994). This method uses oligonucleotides with dyes attached at the 5'-terminus and four different fluorescent dye colors that are mixed in a single channel. However, this method is very expensive and more rigorous control of variation of the reagents is required. Another disadvantage is that the fluorophores may be darkened by the excitation of the laser.

The development of direct DNA sequencing was facilitated by the advent of cycle and automated sequencing (Park and Moran, 1994). Cycle sequencing is a combination of

PCR and sequencing where the sequencing is carried out by performing the annealing and extension steps over and over using the same template. This technique requires substantial optimizations. Full or partial automation of DNA sequencing is possible as a result of the repetitious nature of DNA sequencing. The automated DNA sequencing can be done either by robotics or temperature cycling devices (Oste 1989).

1.8. Aims of study

The lizardfish form a significant component of the by-catch from the prawn trawl fisheries across northern Australia. The dominant species are *Saurida undosquamis* and *Saurida* sp2, morphologically very similar and as such often incorrectly identified or simply grouped together as one or the other. *Saurida* sp2 may be synonimous with *S. grandisquamis,* research in progress (Last, pers. Comm.).

The aims of my study were (i) to clarify the specific status of *S. undosquamis* and the cohabiting species, *Saurida* sp2 (ii) to study the population genetics of both species from the North West Shelf of Australia, the Gulf of Carpentaria and Queensland coast, and (iii) to conduct a phylogenetic analysis of as many species of *Saurida* as possible with the principal object to clarify the *undosquamis* species complex.

Two molecular techniques have been used in my study, i. e. isozyme electrophoresis and direct sequencing of mitochondrial DNA.

CHAPTER 2. MATERIALS AND METHODS

2.1. Sample collection and storage

Samples of lizardfish were collected from three areas across northern Australia - North West Shelf (NWS), Gulf of Carpentaria (GOC) and east coast of Queensland (QLD) (Fig. 2.1). The NWS and GOC samples were collected by the CSIRO Division of Marine Research as part of their efforts of fish and prawn research programs in these regions. The QLD sample was part of the by-catch from a commercial prawn trawl, and the NSW2 sample was collected by Western Australia Fisheries during a trawl-fish survey. The location, dates and size of samples are given in Table 2.1 and the samples localities were depicted in Figure 2.1. Sample locations were chosen to allow comparison of the saurids throughout their range in Australian waters. The second sample from the North West Shelf was taken to allow an investigation of any temporal variability of results.

Table 2. 1. Location, dates and sample sizes of saurids from northern Australian waters.

Samples	Date	Sample sizes	Localities
North West Shelf 1	September 1995	363	*c.* 19°S 118°E
North West Shelf 2	June 1996	89	*c.* 19°S 118°E
Gulf of Carpentaria	October 1995	147	*c.* 12° 30'S 141° 30'E
Queensland	12 July 1996	181	*c.* 14°S 143° 30'E

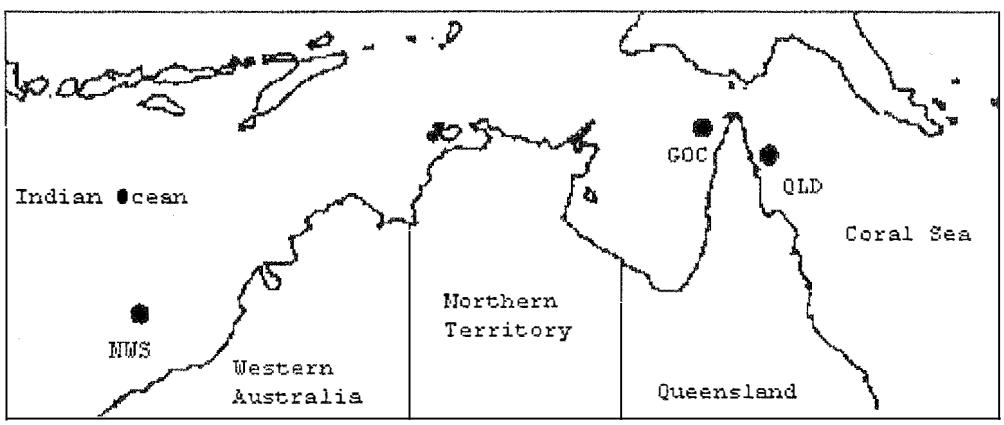


Figure 2.1. Samples localities of *S. undosquamis* and *Saurida* sp2 across northern Australia.

Whole fish were frozen to -30°C on board the sampling vessels. They were transported

frozen to Hobart where they were stored at -80°C until needed.

2.2. Tissue harvesting

The fish were partially thawed in cold running water and small (c.100 mm^3) pieces of liver and muscle were excised and placed in individual plastic storage tubes and quickly refrozen to -80°C. Muscle samples were always taken from the left side of the fish so that the other side could be used for later morphological examination. The carcasses, after individual labelling, were refrozen at -20°C.

2. 3. Isozyme electrophoresis

2. 3a. Sample extraction

About 20 mm^3 of muscle or liver tissue were sliced from the frozen samples for enzyme extraction. Samples were ground in a few drops of distilled water (dH_20) in an Eppendorf tube using a small teflon pestle. The extracts were then centrifuged in the tubes for 2 min at 11 000 rpm and the supernatant was pipetted off directly into wells in the gel plates for electrophoresis. The balance of the extract was frozen in Eppendorf tubes at -80°C for later use.

2. 3b. Electrophoresis

The isozyme electrophoresis was conducted using cellulose acetate plates following the method of Hebert and Beaton (1989). Helena Titan III cellulose acetate plates were run in a custom-made electrophoresis tank. A continuous buffer system was used and, therefore, the gels were pre-soaked in the running buffer for a minimum of 20 min. The soaking buffer was changed as soon as it started to become cloudy with cellulose acetate particles. The running buffers in the gel tanks were replaced fortnightly or after 10 electrophoretic runs.

The cellulose acetate gels were removed from the soaking buffer and blotted dry between sheets of filter paper. A 10 µl aliquot of supernatant (extract) for each sample was pipetted into individual wells of the applicator sample plates. The extracts were applied to the gel using a Super Z-12 applicator kit.. One application to each load zone was sufficient for most enzymes especially the high activity enzymes (e.g. PGM and PGI). For fast migrating enzymes (e.g. PGM), two load zones were applied on the same gel with one zone centrally positioned and the other zone near the cathodal end. In this way, 24 individuals were analyzed on one gel. The 10 µl aliquot per well was sufficient

extract to apply to multiple gels, e.g. for muscle extract, gels were run for PGM, PGI and other enzymes that predominate in muscle and likewise for liver tissue, gels were run for MPI, ADA and other enzymes that predominate in liver. The applicator teeth were cleaned before applications were made with another extract.

Two buffer systems were used, i.e., tris glycine (TG) and tris citrate (TC). The primary difference between these two buffers is their pH. TG has a pH of 8.5, whereas TC has a pH of 7.0. The recipes to make up 1 liter TG were 30 gm Trizma base, 144 gm Glycine and diluted 1:9 TG:water. The recipes for TC were 36.28 g Trizma base, 20.24 g citric acid in 4 liter of water.

The gels were run after placing the acetate side down on the wicks of the electrophoresis tank; care was taken to ensure good contact between the gel plates and the wicks. All enzymes migrated anodally so the application sites were positioned cathodally. Electrophoresis was carried out at 200 V with current flow about 1.5 mA per gel for TG buffer and 150 volts and about 4 mA per gel for TC buffer. Electrophoresis with tris glycine as a buffer system was carried out at room temperature while electrophoresis with tris citrate was run at 4°C. Most enzymes were run at pH=8.0, except AAT that ran at pH=9.0, and CK that ran at pH=7.0 The buffer and enzyme systems are listed in Table 2.3.

2. 3c. Staining systems

The gel staining was carried out at the end of the electrophoretic run to reveal the positions of the proteins. Staining protocols (Table2.4) generally followed Hebert and Beaton (989), Richardson *et al.* (1986), and Morizot and Schmidt (1990) with some modifications developed in the Genetics Laboratory at the CSIRO Division of Marine Research in Hobart. All enzymes, except general protein, were stained by pouring over the gel the staining solution that consisted of a 2 ml mixture of chemical stocks and 2 ml agar. The stain mixtures were prepared while the gel was running except for certain chemicals (PMS and linking enzymes) that were added just before the staining process. Melted agar was added last. Some proteins stained in a short period of time at room temperature (e.g. PGM, PGI and general protein), but others (e.g. MPI, ADA) stained relatively slowly and needed incubation (37° C). The incubation of the plates increased the rate of enzyme reaction and shortened the staining time. Slower staining periods of time may cause diffusion of the enzyme molecules and the reduce sharpness of the

banding patterns (Brewer, 1970).

General protein staining was achieved by immersing the gels in a brilliant blue solution. After the bands were revealed, the staining solution was removed and the gels were soaked in a fixer for 20 min. The fixer contained a mixture of distilled water (dH_2O), methanol and acetic acid glacial.

2. 3d. Gel scoring and storage

Gel scoring was done with careful and continuous observation after the staining solution was poured onto the gel plates. For high activity enzymes (e.g. PGM and PGI) scoring was quickly followed by drawing a diagram of the banding patterns observed. The appearance, position and intensity of bands were recorded.

All gels, once scored, were retained to permit a re-examination of gel scoring. This was done by removing the agar overlay, rinsing under cold running water, and drying in an oven at approximately 37°C.

Table 2. 2. The buffer and enzyme systems employed in this study.
Tissue: l, liver; m, muscle. Buffer system: TG, tris glycine; TC, tris citrate

Enzyme	Locus	Quaternary structure	EC No.	Tissue	Buffer system	Running time (min)
Adenosine deaminase	*ADA**	Monomer	3.5.4.4	l	TG	30'
Aspartate aminotransferase	*mAAT**	Dimer	2.6.1.1	l	TC	90'
	*sAAT**			l	TC	90'
Creatine kinase	*CK**	Dimer	2.7.3.2	m	TG	45'
Fumarate hydratase	*FH**	Tetramer	4.2.1.2	l	TG	40'
Glucose-6-phosphate isomerase	*GPI-1**	Dimer	5.3.1.9	m	TG	40'
	*GPI-2**			m	TG	40'
L-Lactate dehydrogenase	*LDH**	Tetramer	1.1.1.27	m	TG	30'
Malate dehydrogenase	*MDH**	Dimer	1.1.1.37	l	TG	30'
Malic enzyme	*ME**	Tetramer	1.1.1.40	m	TG	30'
Mannose-6-phosphate	*MPI**	Monomer	5.3.1.8	l	TG	20'
Phosphoglucomutase	*PGM**	Monomer	5.4.2.2	m	TG	20'
Superoxide dismutase	*SOD**	Dimer	1.15.1.1	l	TG	20'

Table 2. 3. Staining solution for each enzyme in this study (Hebert and Beaton, 1989).

Enzyme	Recipe
Adenosine deaminase	1 ml Tris HCl, pH=8.0 Adenosine Sodium arsenate 5 drops MTT 5 drops PMS** 10 µl XOD** 10 µl NP** 2 ml agar
Aspartate aminotransferase	2 ml Tris HCl, pH=9.0 2 mg Pyridoxal 5'phosphate (P5P) 2 mg Cystine sulfinic acid 5 mg a Ketoglutaric 5 drops MTT 5 drops PMS** 2 ml agar
Creatine kinase	0.6 ml Tris HCl, pH 7.0 1.5 ml NADP 1.5 ml ADP and D.glucose soln 5 drops phosphocreatine 5 drops MTT 5 drops PMS** 15 µl hexokinase** 2 ml agar
Fumarate hydratase	1.0 ml Tris HCl, pH=7.0 1.5 ml NAD 5 drops fumaric acid (adjust to pH=8.0) 5 drops MTT 5 drops PMS** 25 µl MDH** 2 ml agar
Glucose-6-phosphate isomerase	1.0 ml Tris HCl, pH=8.0 0.6 ml NADP 5 drops fructose-6-phosphate 5 drops MTT 5 drops PMS** 10 µl G6PDH** 2 ml agar
Lactate Dehydrogenase	1.0 ml Tris HCl, pH=8.0 1.5 ml NAD 2 drops DL-Lactic acid 5 drops MTT 5 drops PMS** 2 ml agar

Enzyme	Recipe	
Malate dehydrogenase	1.0 ml Tris HCl pH=8.0 1.5 ml NAD 13 drops Malic substrate 5 drops MTT 5 drops PMS** 2 ml agar	
	Malic substrate	180 ml water 20 Tris HCl Ph=9.0 3.68 gm L-Malic acid adjust to Ph=8.0
Malic enzyme	0.6 ml Tris HCl, pH=8.0 1.5 ml NADP 12 drops Malic substrate 2 drops $MgCl_2$ 5 drops MTT 5 drops PMS** 2 ml agar	
Mannose-6-phosphate isomerase	1.0 ml Tris HCl, pH=8.0 1 ml NADP 5 drops D-mannose-6-phosphate 5 drops MTT 5 drops PMS** 5 µl PGI** 10 µl G6PDH** 2 ml agar	
Phosphoglucomutase	1.0 ml Tris HCl, pH=8.0 1.5 ml NADP 5 drops $MgCl_2$ 5 drops Glucose-1-phosphate 5 drops MTT 5 drops PMS** 10 µl G6PDH** 2 ml agar	
Superoxide dismutase	1.0 ml Tris HCl, pH=8.0 5 drops MTT 5 drops PMS** 2 ml agar	

** = pour just before staining

2. 3e. Nomenclature

Loci and alleles were designated by the nomenclature outlined in Shaklee *et al.* (1990). Multiple loci were numbered according to the extent of migration in cellulose acetate support medium with '1' representing the locus with the fastest mobility towards the anode. Alleles within each locus were designated by the electrophoretic mobilities of their product relative to the electrophoretic mobility of the most common allele (100) in *S. undosquamis* from North West Shelf. The calculation of the relative mobilities was

rounded to the nearest 5%.

2.4. DNA extraction and sequencing

2. 4a. CTAB extraction protocol

Total DNA was extracted using the modified CTAB protocol described by Grewe *et al.* (1993).

Frozen (up to 100 mg) white muscle tissue was homogenised by grinding in 600 µl of CTAB buffer with a plastic pestle. Five micro litres of 20 $mg.ml^{-1}$ proteinase K was added and samples were incubated at 65° C for 60 min. The homogenate was extracted with 600 µl of chloroform iso-amyl alcohol (24:1) and centrifuged at 13 000 rpm for 20 min. The supernatant was pipetted off and mixed with 600 µl of phenol-chloroform-iso-amyl alcohol (25:24:1) and centrifuged at 13 000 rpm for 10 min. This step was repeated twice. The upper aqueous layer was taken off and mixed with 600 µl of chloroform iso-amyl alcohol (24:1). The upper aqueous layer was transferred to a new tube with 700 µl of cold (-20° C) iso-propanol. DNA was allowed to precipitated overnight at -20° C.

The tube was centrifuged at 13 000 rpm for 20 min at room temperature. The supernatant was pipetted off and 500 µl of 70 % ETOH was added and the samples were centrifuged at 13 000 rpm for 10 min. The ethanol was removed and the DNA pellet was dried under vacuum for about 30 min. The pellet was resuspended in 50 µl of dH_2O. The DNA pellet was allowed to rehydrate for several hours at 4°C prior to using the DNA in a PCR experiment.

2. 4b. Amplification of mtDNA

The Polymerase Chain Reaction (PCR) protocol for amplification of mtDNA was adapted from the method described in Palumbi *et al.* (1991). Double stranded (symmetric) amplifications were directed for the cytochrome *c* oxidase subunit 1 gene (CO1), 12sRNA, and cytochrome *b* by the following primers. For CO1 (Folmer *et al.*, 1994), the primers were HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'). For 12s RNA, the primers were 12sai-L (5'-AAACTAGGATTAGATACCCTATTAT-3') and 12sbi-H (5'-AAGAGCGACGGGCGATGTGT-3'). For cytochrome *b*, the primers were CB1-L (5'-CCATCCAACATCTCAGCATGATGAAA-3') and CB2-H (5'-

CCCTCAGAATGATATTTGTCCTCA-3').

Double stranded amplifications for cytochrome c oxidase and 12s RNA were basically performed in the same conditions, except for the primers. Fifty microlitre volumes containing 31.8 µl H_2O, 5 µl 10x buffer, 3.5 µl $MgCl_2$ (25mM), 0.5 µl dNTP (25mM), 5 µl BSA (10x), 0.2 µl Taq polymerase, 2 µl DNA (with no dilution) were used. For cytochrome c oxidase, 1 µl HCO2198 primer (10mM) and 1 µl LCO1490 primer (10mM) were added. For 12s RNA, 1 µl 12sai-L (10mM) and 1 µl 12sbi-H (10mM) were added..

PCR conditions for the double stranded amplification of cytochrome *b* were slightly different from the above conditions. The differences were in the amount of DNA that varied from 2 - 4 µl (with 1:10 dilution and without dilution), the use of 4 µl $MgCl_2$ (25mM) and 0.3 µl Taq polymerase. The amount of H_2O was adjusted to make up the total to 50 µl solution.

The PCR cycle parameters for double-stranded PCR were a modification of those described by Folmer *et al.* (1994). For cytochrome *c* oxidase the profile was one cycle, at 95° C for 4 min, 60° C for 45 sec and 72° C for 2 min, followed by 35 cycles of denaturation at 95° C (30 sec), annealing at 45° C (60 sec), and extension at 72° C (90 sec) and one final extension at 72° C for 5 min. For 12s RNA the profile was, one cycle, at 94° C for 4 min, followed by 35 cycles of denaturation at 94° C (30 sec), annealing at 58• C (60 sec), and extension at 72° C (90 sec) and one final extension at 72° C for 5 min.

For cytochrome b, a reverse touchdown PCR was used for cycle parameters for double-stranded PCR. The profile was one cycle, at 94° C for 4 min, followed by 5 cycles of a reverse touchdown PCR — start cycle was denaturation at 94° C for 30 sec, annealing at 45° C for 1 min, extension at 72° C for 1 min 30 sec, and the subsequent 4 cycles were carried out by increasing the annealing temperature 1 degree for each cycle. The next step was 40 cycles of denaturation at 94° C for 30 sec, annealing at 50° C for 1 min, and extension at 72° C for 1 min 30 sec. The final extension was at 72° C for 5 min.

2. 4c. Visualising and extracting PCR products

Amplifications were confirmed by visualizing the PCR products on an agarose gel using a BRL 'Horizon 58' apparatus.

Subsamples of the PCR products (5µl) were loaded onto 1% agarose in 1x TBE buffer (working concentration: Tris 0.089, Boric acid 0.089 M, EDTA 0.002M). A DNA ladder (Promega) was used to check the size of the products. A positive control with known DNA products (orange roughy, *Hoplostethus atlanticus*) was included as well as a negative control to check any possible contamination. The PCR products were electrophoresed at approximately 85 V (constant voltage) for 30 min. Following electrophoresis the gel was stained with 5 µl of ethidium bromide (10mg/ml) in approximately 120 ml of running buffer retained from electrophoresis. The gel was stained for 20 min, rinsed in water for up to 5 min and viewed under ultraviolet light (302 nm). The intensity of the products was examined and the possibility of extra bands or primer dimer coming up was checked. The gel was printed with a video printer.

The remainder of the successful PCR product (45 µl) was run in a larger gel (1.4 % agarose) using Hoefer HE99X apparatus. The PCR products were electrophoresed at approximately 127 V (constant voltage) for 1 h. Following staining, the gel bands were excised under ultraviolet light and weighed.

2. 4d. PCR purification and quantification

The PCR product (45 µl) was purified using a Jetquick spin column technique according to the protocol for gel extraction provided by the manufacturer (Genomed). Gel slices (50-160 mg) were transferred into a 1.5 ml tube and for each 100 mg gel slice, a 300 µl of solution L1 ($NaClO_4$, sodium acetate and TBE-solubilizer) was added. The mixtures were solubilized by incubating at 50° C for 15 min and were mixed by flicking the tubes every 3 min. The solution was loaded into a Jetquick spin column that was placed in a 2 ml receiver tube and centrifuged at maximum speed for 1 min. The flowthrough was discarded and the spin column was reinserted into the receiver tube. Solution L2 (ethanol, NaCl, EDTA and Tris/HCl) was reconstituted by adding ethanol (95-100%). Seven hundred microlitres of the reconstituted solution L2 was added into the spin column. The solution L2 was allowed to stand in the column for 5 min and then centrifuged at maximum speed for 1 min. The flowthrough was discarded and the column was replaced in the receiver tube. The column was centrifuge at maximum

speed for 2 min. The Jetquick spin column was placed into a new 1.5 ml microfuge tube. The DNA elution was carried out by adding 30 µl of sterile water (preheated to 65-70° C) onto the center of the silica matrix of the Jetquick spin column. The tube was centrifuged at maximum speed for 2 min.

The amount of the purified PCR product was quantified by measuring the fluorosence using the TKO 100 mini fluorometer (Hoefer) that has a fixed excitation bandpass source (365 nm) and an emission (460 nm) bandpass filter. The instrument was calibrated using a low range assay buffer and a calf thymus DNA standard. Two micro litres of each DNA sample was mixed with 2 ml of assay solution [10 µl H 33258 stock solution; 10 ml 10x TNE (0.2 M NaCl, 10 mM Tris-Cl, 1 mM EDTA pH 7.4; 90 ml distilled filtered water] and measured in ng.μl^{-1}.

2. 4e. mtDNA sequencing

Cycle sequencing was performed on a GeneAmp PCR systems 9600 with the following temperature profile: rapid thermal ramp to 96° C, 96° C for 10 sec, rapid thermal ramp to 50° C, 50° C for 5 sec, rapid thermal ramp to 60° C, 60° C for 4 min (Perkin Elmer, 1995). Single-stranded DNA was generated for direct sequencing using asymmetric PCR either with LCO1490 (cytochrome oxidase) and 12sbi-H (12s RNA) as the limiting primer. Each sequencing reaction was made up in 20 µl.with the following reagents as described in the ABI Prism Dye-Terminator kit manual (Perkin Elmer): 8.0 µl terminator ready reaction mix, 3-10 µl PCR product (c. 40-45 ng.μl^{-1} amplified DNA template), 0.64 µl LCO1490 primer 5mM (or 12sbi-H primer), volume adjusted with dH_2O. The extension products were purified and precipitated using 3 M sodium acetate, pH 4.6; 95 % and 70 % ethanol. The DNA pellet was dried in a vacuum centrifuge. The extension products were visualised on an AB1I 337 automated sequencer.

2. 5. Allozyme data analysis

BIOSYS-1 release 1.7 (Swofford and Selander, 1989) was used to analyze electrophoretically detectable allelic variation for population genetics studies. Genotype frequency data was used as input for calculation of gene frequencies and conformance to Hardy-Weinberg equilibrium.

The null hypothesis to be tested was that there was no significant difference between samples of each species from the Northwest Shelf of Australia, the Gulf of Carpentaria

and the Queensland coast.

The principle of Hardy-Weinberg equilibrium is that the allele frequencies remain constant from generation to generation. This principle is expected when gametes associate entirely at random, allele frequencies are the same in both sexes, selection, mutation and migration do not occur, and the population is sufficiently large.

Generally, there are three kinds of tests for comparing observed genotype frequencies to those expected from Hardy-Weinberg equilibrium: (i) exact tests, (ii) goodness-of-fit χ^2 tests, (iii) likelihood-ratio tests and log-linear models (Lessios, 1992). The goodness-of-fit χ^2 tests were chosen as they are computationally simple. However, there is a major disadvantage with the tests since they are severely affected by small expected frequencies, suggesting the need to pool some genotype classes. For rare alleles, pooling was done so that expected numbers of genotypes in a given class were always greater than one. Pooling the genotypes was done when there were two or more alleles at a locus. There are three classes resulting from pooling the genotype in BIOSYS-1: (i) homozygotes for the most common allele, (ii) heterozygotes for the most common allele with other alleles, (iii) all other genotypes. The results of chi-square analysis after pooling are used with one degree of freedom. As a consequence of pooling of rare alleles, this approach may obscure heterogeneity and has the potential of reducing the power of the statistical test. Also, the test is only an approximation of the probability of finding the observed genotype frequencies within the sample.

Another program from Zaykin and Pudovkin (1991), CHIHW, was also used to compare the result of the goodness-of-fit χ^2 tests from BYOSIS. The program estimates their significance using conventional chi-square test and pseudoprobability test. It estimates the probability of null-hypothesis or agreement with Hardy-Weinberg using the Monte Carlo procedure of Roff and Bentzen (1990). This procedure obviates the need to pool rare alleles. A total of 1000 randomizations of the data were performed, with the significance of the result being the number of times each of the randomized replicates was greater or equal to the observed value, divided by 1000.

The extent of genetic differentiation among samples was estimated using Nei's (1973) gene diversity (G_{ST}) statistics. G_{ST} is equal to $(H_T - H_S)/H_T$, where H_T is the total heterozygosity or genetic diversity and H_S is the mean genetic diversity per sample. G_{ST}

was estimated from the mean of the H_T and H_S values across all loci. The range of expected G_{ST} values is from 0 when samples share the same alleles at the same frequencies to 1 for samples that share no alleles. This value represents the proportion of total genetic diversity that can be attributed to differences between samples, e.g. for a G_{ST} value of 0.1 in a locus, 10% of the observed variation in allele frequencies is attributable to variation between samples and 90% to variation within samples.

The magnitude of G_{ST} that could be attributed to sampling error alone was estimated using a bootstrapping procedure (Elliott and Ward, 1992). The method determines if the observed G_{ST} values are due to sampling error or population heterogeneity. A mean value of the magnitude of G_{ST} (termed $G_{ST\text{-null}}$) was estimated for each locus from 1000 bootstrap replications of the data set. The number of times the bootstrap $G_{ST\text{-null}}$ is greater than or equal to the observed G_{ST} was estimated. This equals the probability that the observed value occurred due to sampling of the populations i.e. if the bootstrap values that are greater than the observed G_{ST} are <5%, the confidence level that G_{ST} is due to population differentiation is 95%.

Two kinds of techniques were used to perform homogeneity tests among areas with χ^2 contingency tests of allele numbers. These are the standard procedure, BIOSYS-1 from Swofford and Selander (1989) and CHIRXC from Zaykin and Pudovkin (1991) that uses a Monte Carlo simulation as suggested by Roff and Bentzen (1990). While the former requires rare alleles to be pooled, the latter obviates the need to pool rare alleles. In the Monte Carlo procedure, the number of randomizations of the original data set is used to test the accuracy of the estimate of a (i.e. a type I error). A large number of randomizations of the data set is needed to obtain the expected distribution of χ^2. In this study, 1000 randomizations of the data were generated. The probability of obtaining the observed value by chance was measured. This was done by dividing the number of times each of the randomized replicates was greater or equal to the observed value, by 1000. The hypothesis that there is no geographic variation was tested under 0.05 significance level.

To avoid the error that significance for deviations may be due to chance, it is important to adjust significance levels for the number of tests carried out. Several methods for adjusting significance levels for multiple tests are available (Lessios, 1992). These include the standard Bonferroni and the more powerful technique, sequential Bonferroni

(Hochberg, 1988), that works by ordering tests according to their probability value. In this study, the former method was used. This method works by dividing the predetermined significance level (α=0.05) by the number of tests (k). If a χ^2 value exceeds the value for α/k, it is considered significant.

Wright's (1943) formula was used to calculate the number of migrants per generation (N_em). The equation is $N_em = 0.25[(1/F_{ST}) - 1]$ where N_e is the effective population size and m is the effective proportion of replacement per generation by immigrants. F_{ST} is equivalent to the relative gene diversity (G_{ST}). If $m<<1$, the relationship between N_em and F_{ST} is approximately true. There are some assumptions underlying this calculation i.e. mutation is ignored, the populations are at demographic equilibrium, and the various genotypes are selectively neutral. Forces that lead to population differentiation in these circumstances are solely due to genetic drift and migration.

2. 6. mtDNA sequence data analyses

Prior to sequence alignment, the sequence data were compared with those in molecular databases to check any possible contamination (exogenous or endogenous). Basic Local Alignment Search Tool (BLAST) was accessed through the National Center for Biotechnology Information on the Internet. BLAST sequence similarity searching and basic BLAST search routines were chosen. The sequence with the highest rank of similarity was used as a reference for sequence alignment.

The sequence data was initially aligned by eye (manual) with the Sequence Navigator program from ABI (Perkin Elmer). Nucleotide positions not associated with any electropherogram peak were checked. Alignment was obtained by inserting gaps into one or more positions in the sequence that was thought to be homologous with the same column of the data matrix. The gaps correspond to insertions or deletions. Regions of the sequence data were eliminated from the analysis if the regions were very divergent such that it could not be aligned by manual methods. This method may seem crude and ineffective with arbitrary discarding of data, however, it often produces optimal alignments by demonstrating potential homology between regions of two different molecules. In the case that the assumption of homology has been incorrectly inferred, the consequences are far less severe in the study of similarity than in the phylogenetic reconstructions (Swofford and Olsen, 1990).

In addition to manual alignment, a computerized alignment algorithm was used to simplify the task of aligning sequences. Basically, there are two kinds of alignment i.e. comparative and multiple alignments. Clustal alignment (under the Sequence Navigator program) was chosen to perform multiple sequence alignment. One of the advantages of this method is that multiple sequences are aligned with a good balance between speed and performance. The sequences being aligned are assumed to have an evolutionary relationship. In this method, an alignment algorithm called FAST was used to generate similarity scores for the sequences. The sequences were ordered according to the similarity scores and the resulting tree was used to perform progressive pairwise alignment of the sequences.

PAUP 3.1 (Swofford, 1993) was used to construct phylogenetic relationships from discrete character data under the principle of maximum parsimony. For sequence data, each nucleotide position represents a character and the four nucleotide bases represent the possible character-states. The evolutionary steps (changes or transformation from one character state to another) are base substitution; transition or transversion. In parsimony methods, character states were assigned to minimize the total number of changes required by a particular character on a given tree. The optimal tree (most parsimonious) was obtained if the length of the tree was less than or equal to the length of any other possible tree for the same data.

Basically, there are two methods for searching the optimal trees (Swofford and Olsen, 1990) i.e. exact algorithms and heuristic approaches. The former method guarantees the discovery of all optimal trees but is only useful for small to moderate size of data sets (8 to 20 taxa, depending on the criteria). The use of larger data sets in this method is restricted by the amount of computing time. There are two kinds of exact algorithms approaches i.e., exhaustive search (useful for up to 11 taxa) and branch and bound search (may be used for up to 20 taxa). As in the exhaustive search, branch and bound search also identify all optimal trees. However, the later can not be used to generate the frequency distribution of tree lengths as in the former method.

In this study, the branch and bound method was used to analyze cytochrome oxidase *c* subunit 1 gene and in the three genes combined (CO1, Cytochrome *b,* and 12S RNA). Heuristic approach was used to analyze Cytochrome *b* and 12S RNA.

There are two basic options to evaluate the limitation of heuristic approaches i.e. using stepwise addition and branch swapping. In stepwise addition, taxa were connected one at a time to a developing tree until all taxa had been placed. An initial tree was developed by using three taxa at the beginning. The addition sequence was done with the *simple* option in which the distance between each taxon and a reference taxon was calculated. In this procedure, one of the remaining unplaced taxa was selected according to the order given by its rank in the array of advancement indices. The taxa was joined to the tree along one of its (three) branches. Each of the resulting three trees was evaluated and the one that had optimal length was saved for the next round. Another unplaced taxon was added to one of the five possible branches on the tree saved from the previous round. The shortest three resulting from this addition were saved for the next round. The process was terminated when all taxa were joined to the tree. Stepwise addition generally does not find global optimal trees and is prone to local-optima problems.

The second option, branch swapping, was used to improve the initial estimate by performing sets of predefined rearrangements. This process attempts to find shorter trees with the tree bisection-reconnection (TBR) algorithm option. In this option, two disjoint subtrees were produced by bisecting the tree along a branch. A pair of branches, one from each subtree, was joined to reconnect the subtrees. Evaluation was carried out on all possible bisections and pairwise reconnections. Other options that were effected were Mulpars and steepest descent. The former option requested that all of the equally most parsimonious trees were saved. The latter requested that the round continued even if a shorter tree was found until all trees from the previous round had been examined. The tree(s) that gave the most improvement for the next round was used.

For large data sets that took up vast amounts of computer time and without resulting in any new trees when trying further rearrangements, even with heuristic search, the following solutions were carried out. The option of collapsing zero-length branches and the option of keeping "near minimal trees" were chosen. In the former option, PAUP abandoned the current round if it found a tree that has been found in a previous round. Branch swapping in the previous round was assumed to find all trees in the island to which that tree belongs. Since the assumption may not be true, the resulting trees were not necessarily all of the minimal trees. In the latter option, trees were saved by specifying length (less or equal).

There are three possible kinds of characters based on the information they provide. The first type of characters is that which is both informative and reliable since it provides the truth about the past. The second type is that which may be reliable but uninformative. The third type is that which is uninformative. One way to identify the unreliable characters is by assigning weight to the characters. Lower weight is assigned to characters that either violate the assumptions of a method or make the method inconsistent.

Generally, there are two methods for assessing the confidence interval in phylogenetic analysis i.e. the jacknife and the bootstrap. While the former provides little information for the distribution of the estimates, the latter can provide many estimates to give good approximation to the distribution of the original estimator. In this thesis, the bootstrap method that follows Felsenstein (1985) was chosen.

The bootstrap method that is under PAUP was used to assess the repeatability of a given result and to measure the probability that a phylogenetic estimate represents the true phylogeny. The method works by drawing random samples of the same size as the original data set with replacement. Each data unit has an equal chance of being chosen at any stage. Each of the bootstrap replicates was analyzed and the results were used to indicate the error involved in making estimates from the original data. For phylogenetic estimates, the original species was kept while sampling characters with replacement. The assumptions that underlie the process were that the character has been drawn and evolved independently. All of the bootstrap trees were subjected to a majority-rule consensus that assigns that groups must be present in over half of the derived trees. The confidence level of a group appears as a percentage of the bootstrap trees. Differences between groups were considered to be significant if showing 95% or more of the bootstrap value.

Another program, DNADIST application of the PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, 1993), was used to correct pairwise sequence distance for multiple substitutions using the Kimura two-parameter model (Kimura, 1980) with a transition-transversion ratio of 2. 0.

CHAPTER 3 RESULTS

3. 1. Isozyme data analyses

3. 1a. Enzyme loci and species identification

Initially, general proteins and 18 enzymes representing 21 gene loci were screened by cellulose acetate gel electrophoresis to examine resolution of banding patterns. This screening was used to identify monomorphic loci for species identification and polymorphic loci for population studies. Eleven enzymes representing 13 presumptive loci had sufficient activity and could be scored reliably (Table 2. 2). Other presumptive loci were poorly resolved; these were *IDH*, ESTD*, ADH*, ALD*, G6PDH*, GPDH** and *PEP**. Four different substrates were used in *PEP** staining, i.e., Glycyl-L-Leucine, Leu-Leu-Leu, Phe-Leu, L-Leucylglycyl-Glycine.

From the initial screening, general protein assay and *LDH** were chosen to delineate presumptive taxa of saurids from the North West Shelf, Gulf of Carpentaria and Queensland coast. A total of 779 fish, all provisionally identified when collected as *S. undosquamis*, *Saurida* sp2, *S. longimanus* or saurids, was screened. General proteins were clearly and consistently resolved in all samples. However, the banding patterns of the electromorphs were complicated and interpretation was confusing. Initially, the general protein patterns were classified into four groups. The assumption that underlaid these groupings was that some patterns in the electromorphs represented polymorphic loci (Figure 3.1). Later, this assumption was proved to be wrong in group III. Morphological (P.R Last, pers. comm) and mtDNA data showed that there were two distinct species in this group. So, five, instead of four groups were accepted. These were associated with the following species, (I) *Saurida* sp2, maybe synonymous with *S. grandisquamis*, research in progress (Last, pers. comm.); (II) *S. undosquamis* Richardson, 1848, (III) *S.* cf *argentea* Macleay, 1882; (IV) *S. filamentosa* Ogilby, 1910, (V) *S. longimanus* Norman, 1939. One of the bands revealed by the general protein staining was confirmed, by specific staining, to represent the *CK** locus. Three electromorphs were detected for this locus; these are illustrated in Fig. 3.1. Other enzymes were not used for checking the general protein patterns.

In the case of *LDH**, there were two forms of electromorph with very strong bands (Figure 3.2). These bands tended to smear quickly within a few minutes after staining. The two forms of the electromorph, fast and slow, referred to its electrophoretic mobility. *LDH** was monomorphic in almost all samples of saurids. Only one out of 779 fish had a heterozygote locus with a distinct 5 banding pattern; this accords to the known subunit number for this locus.

The electromorph patterns for general proteins, *CK** and *LDH** could be married with the morphotypes of known species (Table 3.1). Genetic and collection ID of saurids across northern Australia are given in Table 3. 2.

Other fixed allelic differences between *Saurida* sp2 and *S. undosquamis* were found subsequently for the *SOD**, *MDH**, and *AAT-2** loci (Table 2.3).

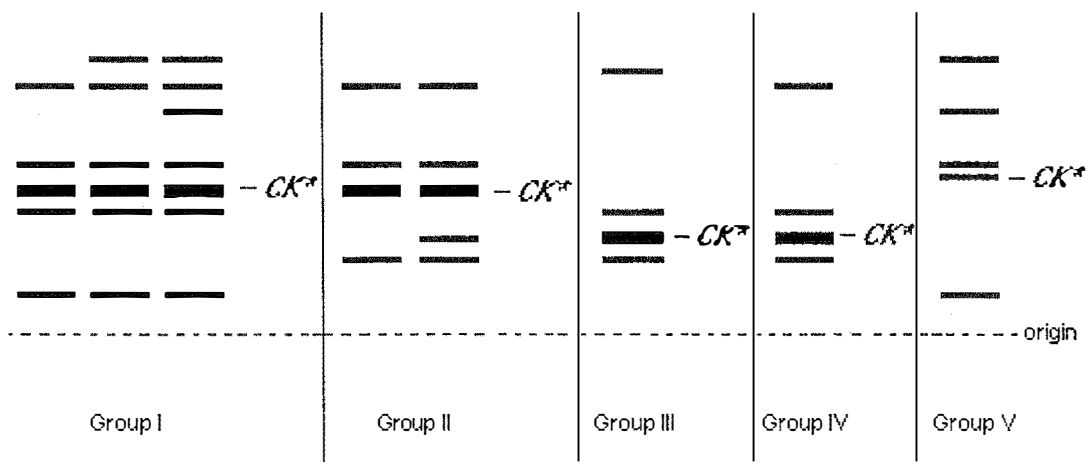


Figure 3. 1. General protein patterns and the corresponding *CK locus [Group I = *Saurida* sp2; Group II = *S. undosquamis* Richardson 1848; Group III = *S.* cf. *argentea* Macleay, 1882; Group IV = *S. filamentosa* Ogilby, 1910; Group V = *S. longimanus* Norman, 1939]; initially, Group III and IV were assumed to be one group.**

Figure 3. 2. Two forms of *LDH with very strong bands; slow = *S. undosquamis* Richardson, 1848; *S.* cf. *argentea,* Macleay, 1882; *S. filamentosa* Ogilby, 1910; fast = *Saurida* sp2; *S. longimanus* Norman, 1939.**

Table 3.1. Electromorph patterns for general proteins, *LDH and *CK** for five species of saurids from northern Australian waters; see Figs 3.1 and 3.2 for patterns.**

	General protein group	*CK** slow (S), medium (M), fast (F)	*LDH** fast (F), slow (S)
Saurida sp2	I	M	F
S. undosquamis	II	M	S
S. cf *argentea*	III	S	S
S. filamentosa	IV	S	S
S. longimanus	V	F	F

Table 3. 2. Genetic and collection ID of saurids across northern Australia.

Location	Genetic ID	Number of fish	Collection ID	Number of Fish
North West Shelf 1	*Saurida* sp2	226	*Saurida* sp2	176
	S. undosquamis	98	*Saurida* sp2 and *S. longimanus*	67
	S. longimanus	39	*S. undosquamis*	55
			Saurida ?	65
North West Shelf 2	*S. undosquamis*	90	grinners	90
Gulf of Carpentaria	*Saurida* sp2	117	*Saurida* sp2	3
	S. cf *argentea*	16	*S. undosquamis*	8
	S. filamentosa	13	saurids	135
Queensland	*Saurida* sp2	135	saurids	180
	S. undosquamis	31		
	S. cf *argentea*	6		
	S. filamentosa	8		
Total		779		779

3. 1b. Analysis of Saurida sp2 *samples*

A minimum of 22 fish from each of three sampling areas (NWS, GOC, QLD) was used to study the population structure of *Saurida* sp2, with 12 loci (Table 3.3).

Table 3.3. Allele frequencies at 12 loci in *Saurida undosquamis* Richardson, 1848 and *Saurida* sp2 from northern Australian waters.

(n), number of individuals scored for a given locus; – , not detected.

Locus, allele	*S. undosquamis*			*Saurida* sp2		
	North West Shelf of Australia 1	North West Shelf of Australia 2	Queensland	North West Shelf of Australia	Gulf of Carpentaria	Queensland
*MPI**						
(n)	(92)	(89)	(31)	(177)	(115)	(136)
120	–	–	–	0.017	–	0.033
110	0.016	0.017	0.081	0.475	0.909	0.857
100	0.924	0.938	0.919	0.497	0.091	0.110
90	0.060	0.039	–	0.011	–	–
80	–	0.006	–	–	–	–
*PGM**						
(n)	(24)	(24)	(31)	(211)	(116)	(136)
190	–	–	–	–	–	0.004
145	–	–	0.065	0.118	0.134	0.125
100	1.000	1.000	0.919	0.874	0.866	0.860
90	–	–	0.016	0.002	–	–
55	–	–	–	0.005	–	0.011
*GPI-1**						
(n)	(91)	(85)	(24)	(24)	(24)	(24)
100	1.000	1.000	1.000	1.000	1.000	1.000
*GPI-2**						
(n)	(83)	(85)	(24)	(24)	(24)	(24)
135	0.343	0.376	–	–	–	–
100	0.651	0.624	1.000	1.000	1.000	1.000
70	0.006	–	–	–	–	–
*MDH**						
(n)	(83)	(84)	(40)	(23)	(22)	(22)
265	–	0.077	0.213	–	–	–
100	0.964	0.875	0.712	1.000	1.000	0.977
0	0.036	0.048	0.075	–	–	0.023
*ADA**						
				(174)	(114)	(136)
				0.032	0.018	0.015
				0.083	0.140	0.088
				0.782	0.711	0.724
				0.046	0.070	0.099
				0.040	0.053	0.070
				0.017	0.009	0.004
*AAT-1**						
(n)	(24)	(23)	(24)	(24)	(114)	(23)
100	1.000	1.000	1.000	1.000	0.987	1.000
65	–	–	–	–	0.013	–

*AAT-2**						
(n)	(24)	(23)	(24)	(24)	(116)	(23)
135	—	—	—	1.000	0.978	1.000
100	1.000	1.000	1.000	—	0.022	—
*FH**						
(n)	(22)	(17)	(23)	(24)	(24)	(24)
100	1.000	1.000	1.000	0.979	0.979	1.000
85	—	—	—	0.021	0.021	—
*LDH**						
(n)	(98)	(90)	(31)	(225)	(116)	(136)
300	—	—	—	1.000	1.000	1.000
100	1.000	0.994	1.000	—	—	—
-10	—	0 .006	—	—	—	—
*ME**						
(n)	(24)	(24)	(24)	(24)	(24)	(24)
100	1.000	1.000	1.000	1.000	1.000	1.000
*SOD**						
(n)	(84)	(89)	(31)	(150)	(101)	(119)
140	—	—	—	0.957	0.946	0.962
100	1.000	1.000	1.000	0.043	0.054	0.038

Four loci showing a high degree of variation were examined in over 100 fish from each sample, for three of these, the most common allele had a frequency less than 0.95: *MPI**, *PGM** and *ADA**. Other presumptive loci showed more limited variation; e.g. *SOD** was polymorphic only in Gulf of Carpentaria. Under the 0.99 criterion, *SOD** was also polymorphic in North West Shelf and Queensland samples. Under the later criterion, *MDH** was polymorphic in Queensland fish, while *AAT-1** and *AAT-2** were polymorphic in Gulf of Carpentaria fish, and *FH** was polymorphic in both North West Shelf and Gulf of Carpentaria fish. The other presumptive loci (*GPI-1**, *GPI-2**, *LDH**, *ME**) were monomorphic in all three samples. All heterozygote banding patterns accorded to the known subunit numbers except in *FH**. Heterozygotes were clearly two banded although *FH** is a tetrameric enzyme. Allele frequencies and sample sizes at each locus for the three samples are given in Table 3. 3.

The direct count estimate of the heterozygosity (H_T) per locus for the 12 presumptive loci ranged from 0% to 45.8%. *ADA** was the most heterozygous locus in samples from the Gulf of Carpentaria (40.4%) and Queensland (39%), while *MPI** was the most heterozygous locus (45.8%) in samples from North West Shelf. Mean heterozygosity (H_T) per area from the direct count ranged from 8.3% to 9.2%. Percentage of polymorphic loci per area ranged from

25% to 33.3% with the $P_{0.95}$ criterion and from 41.7% to 58.3% with the $P_{0.99}$ criterion. A summary of the genetic variability at 12 loci in all samples is given in Table 3. 4.
The results of the chi-square testing for deviation from Hardy-Weinberg genotypic proportions are given in Table 3. 5. For the initial test (BIOSYS), involving the pooling of rare alleles when more than two alleles were present, all chi-square tests had d. f., there were significant deviations at the *MPI** locus (0.027) in the North West Shelf sample and the *ADA** locus for both the North West Shelf (0.033) and Queensland (0.013) samples. None of these probabilities were greater than the Bonferroni-adjusted significance level for multiple tests.

Analysis of the data using the CHIHW program of Zaykin and Pudovkin (1991), which does not require the pooling of rare alleles, confirmed that all samples conformed with Hardy-Weinberg equilibrium under the $P<0.05$ criterion (Table 3.5).

Calculation of coefficients for heterozygote deficiency or excess is given in Table 3.6. *ADA** has significant heterozygote deficiency under $P<0.05$ criterion in all three samples with Selander's value (D) = -0.116, -0.137, -0.139 in NWS, GOC, and QLD, respectively. none of the probabilities were significant after applying the Donferrori correction for multiple tests.

The results of the contingency chi-square analyses of homogeneity of allele frequencies at eight loci are given in Table 3.7. The results indicate genetic stock structuring among the three areas sampled. Significant ($P<0.001$) differentiation was detected at the *MPI** locus.

Differences among the three areas sampled were supported by the gene diversity analyses. The *MPI** locus showed high significant differentiation ($P<0.001$) with a G_{ST} equal to 0.187 that was significantly greater than the $G_{ST\text{-null}}$ value of 0.002. The

Table 3. 4. Genetic variability at 12 loci in three samples of *Saurida* sp2 (standard errors in parentheses).

Population	Mean sample size per locus	Mean no. alleles per locus	Percentage of loci polymorphic*	Mean heterozygosity	
				Direct count	HW expected**
North West Shelf	92 (24.9)	2.1 (0.5)	25	0.092 (0.042)	0.104 (0.051)
Gulf of Carpentaria	75.8 (13.4)	1.9 (0.4)	33.3	0.085 (0.037)	0.090 (0.041)
Queensland	68.9 (16.3)	2 (0.5)	25	0.083 (0.038)	0.089 (0.043)

* A locus is defined as ploymorphic if the frequency of the most common allele is <0.95.
** Hardy Weinberg unbiased estimate (Nei 1978).

Table 3. 5. Chi-square test for deviation from Hardy-Weinberg equilibrium in three samples of *Saurida* sp2.

	North West Shelf			Gulf of Carpentaria			Queensland		
Locus	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*
				Chi-square with pooling (Biosys)					
*MPI**	4.916	1	0.027	1.536	1	0.215	0.267	1	0.605
*PGM**	1.186	1	0.276	0.674	1	0.412	1.023	1	0.312
*ADA**	4.544	1	0.033	2.624	1	0.105	6.140	1	0.013
*MDH**	-	-	-	-	-	-	0.000	1	1
*SOD**	2.273	1	0.132	0.303	1	0.582	0.163	1	0.687
*FUM**	0.000	1	1	0.000	1	1	-	-	-
*AAT-1**	-	-	-	0.013	1	0.908	-	-	-
*AAT-2**	-	-	-	0.045	1	0.832	-	-	-
				Chi-square without pooling (Monte Carlo)					
*MPI**	11.295	-	0.124	1.370	-	0.260	1.440	-	0.748
*PGM**	2.156	-	0.529	0.738	-	0.468	1.901	-	0.486
*ADA**	27.363	-	0.075	19.573	-	0.142	15.73	-	0.323
*MDH**	-	-	-	-	-	-	0.012	-	1
*SOD**	2.002	-	0.260	0.335	-	1	0.184	-	1
*FUM**	0.011	-	1	0.011	-	1	-	-	-
*AAT-1**	-	-	-	0.020	-	1	-	-	-
*AAT-2**	-	-	-	0.056	-	1	-	-	-

P is no of randomizations ≥ true value

Table 3. 6. *Saurida* sp2. Coefficients for heterozygote deficiency or excess in three samples derived from CHIHW (Zaykin and Pudovkin 1991). D=Selander's value

Locus	NWS			GOC			QLD		
	D	*P*		D	*P*		D	*P*	
		one tail	two tail		one tail	two tail		one tail	two tail
*MPI**	-0.132	0.039	0.079	-0.109	0.225	0.225	0.017	0.541	0.857
*PGM**	-0.079	0.151	0.274	0.080	0.340	0.468	-0.066	0.195	0.445
*ADA**	-0.116	0.013	0.016	-0.137	0.020	0.034	-0.139	0.012	0.013
*FUM**	0.021	1	1	0.021	1	1	-	-	-
*AAT-1**	-	-	-	0.013	0.992	1	-	-	-
*AAT-2**	-	-	-	0.022	0.966	1	-	-	-
*MDH**	-	-	-	-	-	-	0.023	1	1
*SOD**	-0.116	0.260	0.260	0.058	0.761	1	0.039	0.847	1

P - estimated probability of D=0

differences between the G_{ST} and the $G_{ST\text{-null}}$ values for the other 7 loci were not significantly different. The G_{ST} value across all eight polymorphic loci was equal to 0.063 indicating that 6.3% of the total genetic variation was attributable to differentiation between samples and the remaining 93.7% of variation was due to differentiation within samples. The mean G_{ST} value of 0.063 was significantly greater than the mean $G_{ST\text{-null}}$ of 0.003, again suggesting significant genetic structuring among the three regions sampled.

Table 3. 7. *Saurida* sp2. Summary of heterogeneity χ^2 for differences in gene frequencies (Monte Carlo tests) and gene diversity statistics for the eight variable loci among three samples.

	Heterogenity χ^2		Gene diversity tests		
Locus	χ^2	P	Nei's G_{ST}	$G_{ST\text{-null}}$	P^A
*MPI**	184.616	<0.001	0.187	0.002	<0.001
*PGM**	6.800	0.626	<0.001	0.002	0.854
*ADA**	19.971	0.028	0.005	0.002	0.052
*MDH**	2.061	0.673	0.015	0.010	0.346
*AAT-1**	1.249	0.567	0.008	0.009	0.391
*AAT-2**	1.249	0.567	0.009	0.009	0.316
*FUM**	1.014	1	0.007	0.012	0.784
*SOD**	0.729	0.707	0.001	0.003	0.808
Across eight loci			0.063	0.003	<0.001

P^A equals to the proportion of cases where randomized G_{ST} are equal or greater than the observed G_{ST}.

Further χ^2 tests were carried out to identify the geographic source of the genetic differentiation revealed by the contingency χ^2 tests. These were carried out by pairwise comparison of each sample. High significant differentiation (P<0.001) was shown at *MPI** between the North West Shelf and both Gulf of Carpentaria and Queensland samples. The estimated probability of homogeneity of the Gulf of Carpentaria and Queensland samples at *MPI** was 0.015, indicating significant genetic differentiation between *Saurida* sp2 populations in these two areas as well. Under the P=0.05 criteria, the *ADA** locus was significantly different between the North West Shelf and Queensland samples (P=0.014).

The number of migrants per area per generation was calculated using Wright's (1943) equation with the G_{ST} value of 0.063 being used as the F_{ST} value. The results indicated that the net movement of fish would be very low (<4 migrants per area per generation). Care needs to be taken in interpreting this result as it was derived from just three polymorphic loci. However, it is further evidence that there is genetic structuring between *Saurida* sp2 populations across northern Australia.

3. 1c. Analysis of Saurida undosquamis samples

A minimum of 17 fish from each of three samples (NWS 1, NWS 2, and QLD) were analysed for each of 11 loci (Table 3.2). No *S. undosquamis* were detected in the Gulf of Carpentaria sample.

There were some problems in the interpretation of the banding patterns for the electromorphs of the *MDH** and *GPI-2** loci for this species. The confusion in the interpretation of *MDH** arose as in most fish this enzyme has duplicate cytoplasmic loci (Ward *et al.* 1992), often with a common electromorph and a mitochondrial gene product. The possible banding patterns if there were two cytoplasmic loci with interlocus heterodimer are depicted in Figure 3. 3b. I observed four genotypes with two types of heterozygotes (Figure 3. 3a). However, one banding pattern (genotypes ff in Figure 3. 3a) observed did not conform with the theoretical explanation of the existence of two loci. Therefore, one, instead of two loci, has been scored in *MDH**.

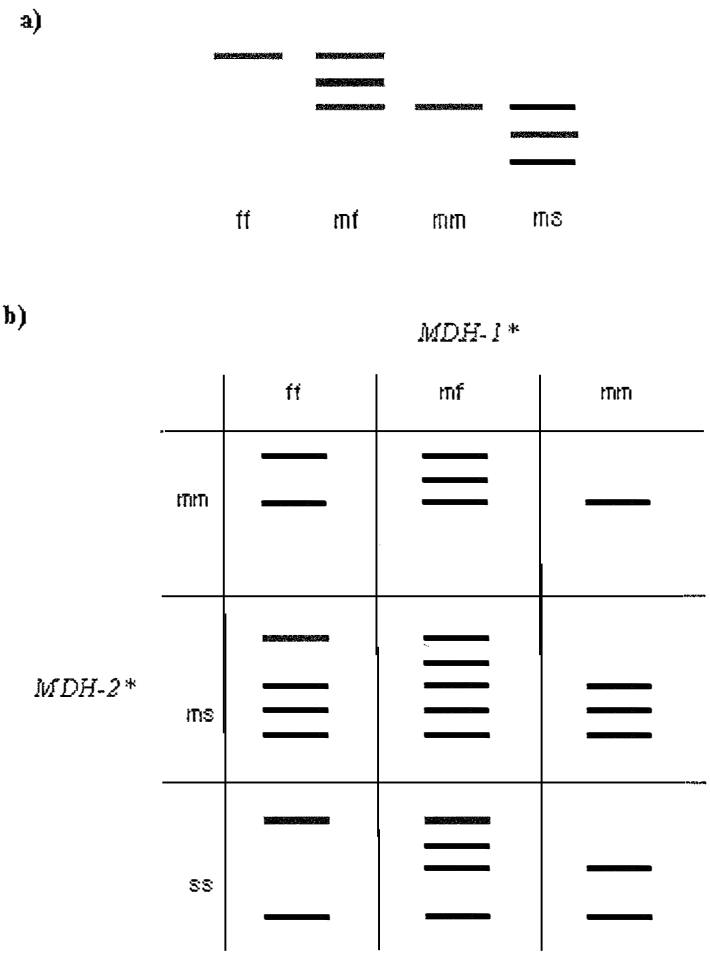


Figure 3. 3. Banding patterns in *MDH, a) observed genotypes, ff suggested the present of a single locus, b) possible banding patterns if two loci, *MDH-1** and *MDH-2**, existed with common mm electromorph.**

For the *GPI-2** locus, the heterozygote was three banded since it is a dimeric enzyme. This locus made interlocus hybrids with the *GPI-1** locus. However, difficulties occurred when some of the banding patterns were different from others, in particular the lack of assumed

interlocus heterodimer bands. The interpretation of the electromorphs is described in Figure 3.4.

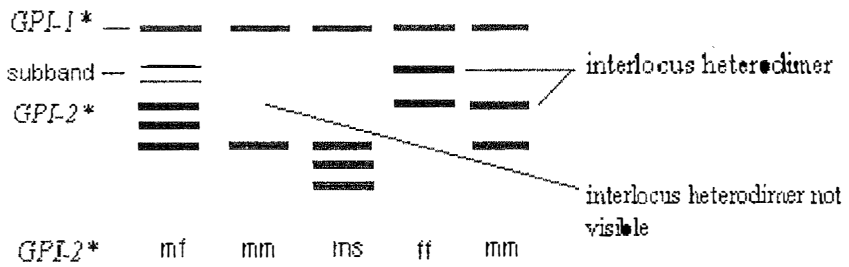


Figure 3.4. *GPI-1 and *GPI-2** patterns and its interpretation (mm = medium, mf = medium fast, ms = medium slow, ff = fast).**

The *ADA** locus was not confidently scored due to enzyme degradation in most of the samples in *S. undosquamis.* So, this locus was removed from the analysis.

The two North West Shelf samples were temporally spaced but from the same area. Heterogeneity χ^2 tests showed that the two samples were different significantly at the *MDH** locus, so they were treated separately. Less than 100 fish was available from each of these samples and the number of fish analysed was further reduced due to enzyme degradation. The Queensland sample had the lowest sample size with a maximum of 31 fish being screened. The low sample size for *Saurida undosquamis* may be relevant to the integrity of the analyses.

Only for *MPI** was the frequencies of the most common allele less than 0.95 in all three samples. The other presumptive loci showed more limited variation. *GPI-2** was polymorphic in the two North West Shelf samples but monomorphic in the Queensland sample. *MDH** was polymorphic under this criterion in the second North West Shelf sample and the Queensland sample. *PGM** was polymorphic only in the sample from Queensland. Under P=0.99 criterion, *MDH** in NWS 1 was also polymorphic. The other presumptive loci (*GPI-1*,ME*, AAT-1*, AAT-2*, LDH*,SOD**) were monomorphic in three samples. Allele frequencies and sample sizes at each locus for the three samples are given in Table 3.2.

The direct count estimate of the heterozygosity (H_T) per locus for the 11 presumptive loci ranged from 0% to 55.4%. Mean heterozygosity (H_T) per area from the direct count ranged

from 6.3% to 8.2%. Percentage of polymorphic loci per area ranged from 18.2% to 27.3% with the $P_{0.95}$ criterion and was 27.3% with the $P_{0.99}$ criterion. A summary of the genetic variability at 11 loci in all populations is given in Table 3.8.

Table 3. 8. Genetic variability at 11 loci in three populations of *Saurida undosquamis* (standard errors in parentheses).

Population	Mean sample size per locus	Mean no. of alleles per locus	Percentage of loci polymorphic *	Mean heterozygosity	
				Direct count	HW expected**
North West Shelf 1	59.0 (10.3)	1.5 (0.2)	18.2	0.071 (0.050)	0.061 (0.062)
North West Shelf 2	57.5 (10.2)	1.6 (0.3)	27.3	0.082 (0.050)	0.075 (0.045)
Queensland	27.9 (1.6)	1.5 (0.2)	27.3	0.063 (0.037)	0.068 (0.042)

* A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95
** Hardy Weinberg unbiased estimate (Nei 1978).

Chi-square testing with and withoutpooling of rare alleles observed indicated there was no significant deviation from Hardy-Weinberg genotypic proportions any of the three samples. A summary of chi-square test results are given in Table 3.9.

Table 3. 9. Chi-square test for deviation from Hardy-Weinberg equilibrium in three populations of *Saurida undosquamis*.

Locus	North West Shelf of Australia 1			North West Shelf of Australia 2			Queensland		
	χ^2	d.f.	P	χ^2	d.f.	P	χ^2	d.f.	P
				Chi-square with pooling (Biosys)					
*MPI**	0.576	1	0.448	0.349	1	0.555	0.188	1	0.665
*GPI-2**	3.774	1	0.052	0.796	1	0.372	-	-	-
*MDH**	0.097	1	0.756	1.624	1	0.202	0.383	1	0.536
*PGM**	-	-	-	-	-	-	0.188	1	0.665
*LDH**	-	-	-	0.000	1	1	-	-	-
				Chi-square without pooling (Monte Carlo)					
*MPI**	0.624	-	1	0.386	-	1	0.239	-	1
*GPI-2**	4.175	-	0.098	0.895	-	0.379	-	-	-
*MDH**	0.117	-	1	1.714	-	0.557	5.684	-	0.103
*PGM**	-	-	-	-	-	-	0.239	-	1
*LDH**	-	-	-	0.003	-	1	-	-	-

Calculation of coefficients for heterozygote deficiency or excess is given in Table 3. 10. *GPI-2** in NWS 1 had a significant heterozygote excess under P=0.05 criterion.

Table 3. 10. ***Saurida undosquamis.*** **Coefficients for heterozygote deficiency or excess in three samples derived from Zaykin and Pudovkin.**

Locus	NWS 1			NWS 2			QLD		
	D	*P*		D	*P*		D	*P*	
		one tail	two tail		one tail	two tail		one tail	two tail
*MPI**	0.068	0.580	0.890	0.048	0.717	0.855	0.088	0.844	1
*MDH**	0.038	0.903	1	0.106	0.252	0.332	-0.151	0.142	0.269
*GPI-2**	0.208	0.042	0.068	0.103	0.260	0.379	-	-	-
*PGM**	-	-	-	-	-	-	0.073	0.824	1
*LDH**	-	-	-	0.006	1	1	-	-	-

P - estimated probability of D=0

Contingency chi-square analyses of homogeneity of allele frequencies at the five variable loci showed a genetic stock structure among the three samples. Significant ($P<0.001$) differentiation was detected at *GPI-2**, and *MDH** (Table 3.11).

Differences among the three samples were supported by the gene diversity analyses. The *GPI-2**, *MDH** and *PGM** loci all returned G_{ST} values significantly ($P<0.001$) greater than the G_{STnull} value. The G_{ST} value across all 11 loci was 0.098. This value indicated that 9.8% of the total genetic variation was attributable to differentiation between samples and the remaining 90.2% of variation was due to differentiation within samples. The mean G_{ST} value of 0.098 was significantly greater than the mean G_{STnull} of 0.007, again suggesting significant genetic structuring among the three regions sampled. Summary of heterogeneity χ^2 for differences in gene frequencies and genetic diversity analysis are given in Table 3.11.

Table 3. 11. ***Saurida undosquamis.*** **Summary of heterogeneity χ^2 for differences in gene frequencies and gene diversity statistics for the five variable loci among three samples.**

	Heterogenity χ^2			Gene diversity tests	
Locus	χ^2	*P*	Nei's G_{ST}	$G_{ST\text{-}null}$	P^A
*MPI**	13.761	0.027	0.012	0.006	0.104
*PGM**	7.995	0.035	0.046	0.013	<0.001
*GPI-2**	27.097	<0.001	0.159	0.007	<0.001
*MDH**	39.309	<0.001	0.070	0.005	<0.001
*LDH**	1.437	0.534	0.004	0.003	0.376
Across all 11 loci			0.098	0.007	<0.001

P^A equals to the proportion of cases where randomized G_{ST} are equal or greater than the observed G_{ST}.

Chi-squared testing on pairwise comparisons between samples was used to identify the geographic source of the genetic differentiation between areas (Table 3. 12). *MDH** showed high significant differentiation ($P=0.001$) between NWS 1 and NWS 2 samples, ($P<0.001$) between NWS 1 and QLD samples, and ($P=0.005$) between NWS 2 and QLD samples. *GPI-2** showed high significant differentiation ($P<0.001$) between NWS 1 and QLD, and between NWS 2 and QLD samples. *MPI** locus showed high significant differences ($P=0.005$)

between samples from NWS 1 and QLD. Under P=0.05, *MPI** also showed significant differentiation between samples from NWS 2 and QLD.

Table 3. 12. Probability of homogeneity from pairwise comparison between samples in *S. undosquamis*.

		NWS 2			QLD		
		*MDH**	*GPI-2**	*MPI**	*MDH**	*GPI-2**	*MPI**
NWS 1	*MDH**	0.001			<0.001		
	*GPI-2**		0.565			<0.001	
	*MPI**			0.708			0.005
NWS 2	*MDH**				0.005		
	*GPI-2**					<0.001	
	*MPI**						0.024

The mean G_{ST} value of 0.382 that was equal to F_{ST} value was used to calculate the number of migrants per area per generation using Wright's (1943) equation. As in the previous analysis on *Saurida* sp2, the results indicate a limited exchange among samples examined of < 1 migrants per area per generation.

3. 2. mtDNA sequence data analysis

3. 2a. Comparison of mitochondrial cytochrome c oxidase subunit 1 gene.

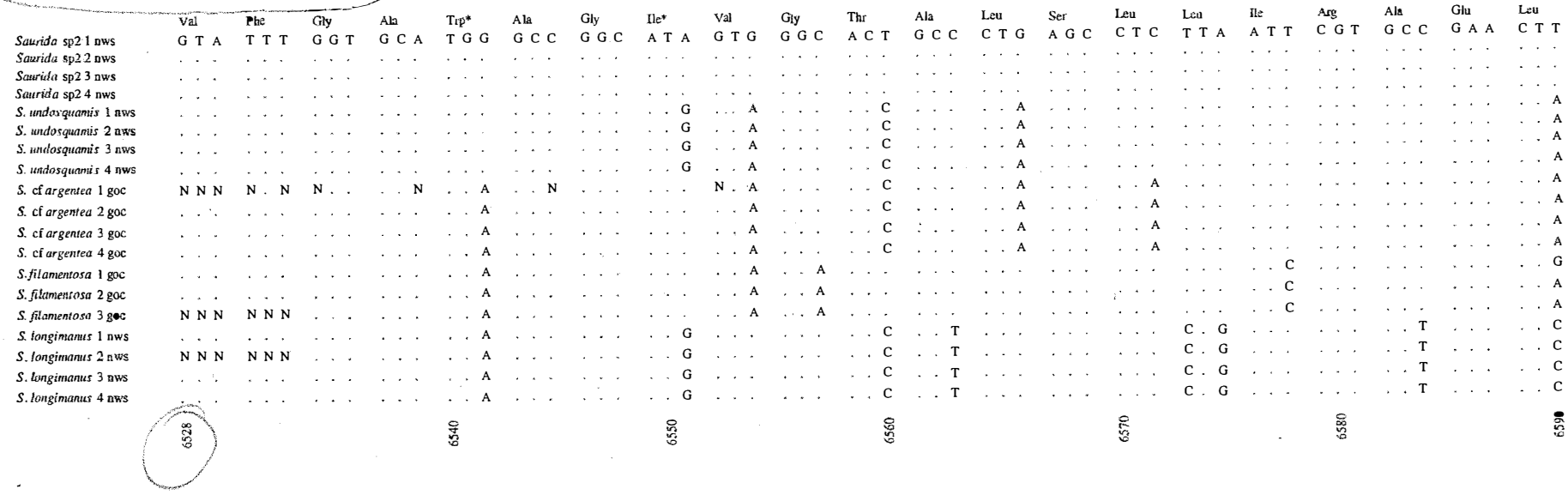
The light strand of the mitochondrial cytochrome oxidase c subunit I gene (COI) was sequenced in five species of saurids . The sequence identity was confirmed from four individuals per species with the exception of *S. filamentosa* where three individuals were used.

It was not possible to obtain samples of all five species from each locality. Therefore samples of *S. undosquamis*, *Saurida* sp2 and *S. longimanus* from the Northwest Shelf were used along with samples of *S.* cf *argentea* and *S. filamentosa* from the Gulf of Carpentaria.

Samples of *S. undosquamis* from Queensland were to be included for sequencing to determine levels of geographic variation however, no PCR product was observed in these individuals.

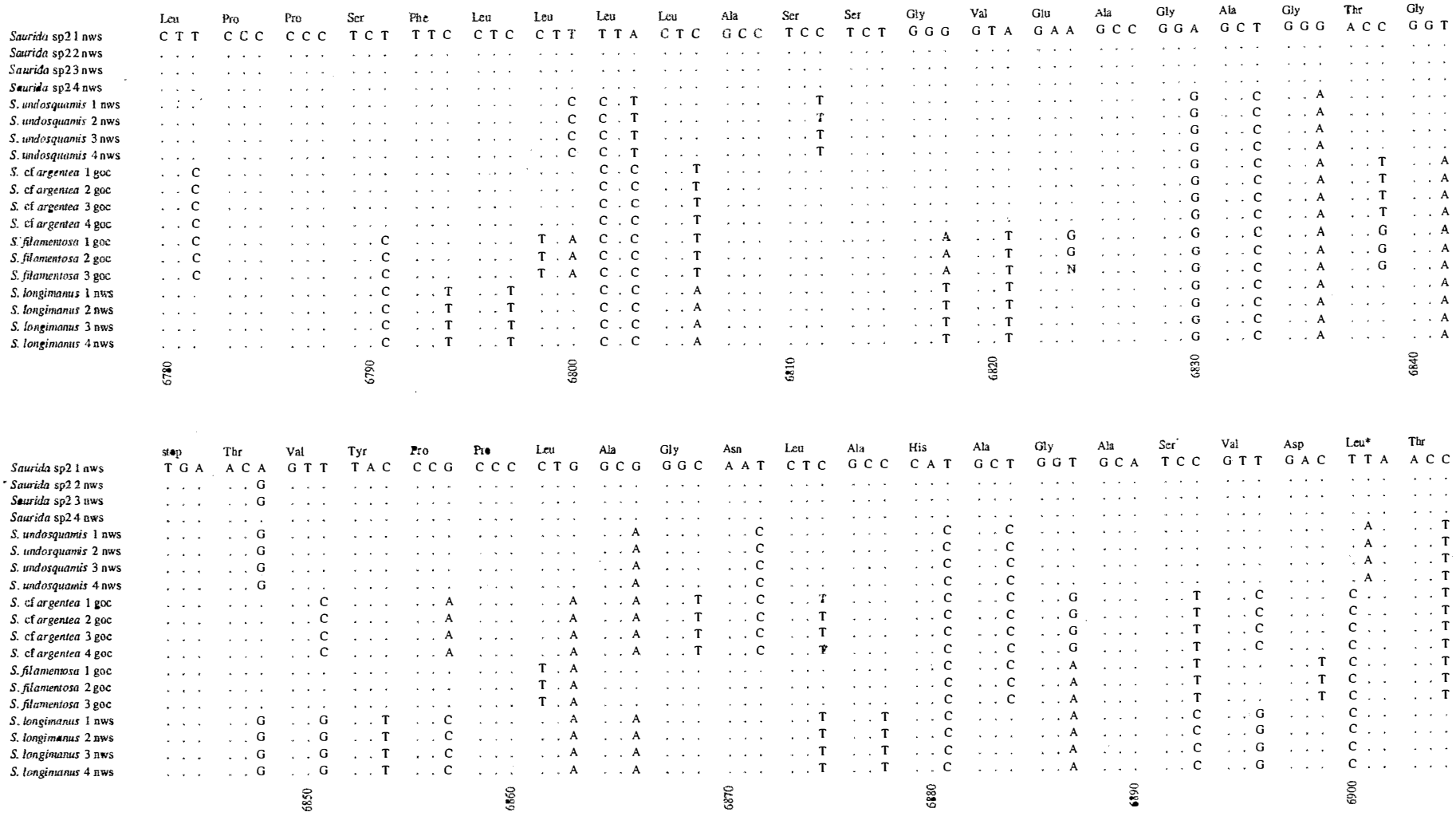
The sequenced COI fragment was 546 base pairs (bp) in length and codes for 182 amino acids (Figure 3.5). The saurid sequence was aligned with the rainbow trout *(Oncorhynchus mykiss)* COI sequence, which was found to be the closest match from a BLAST search of the

Figure 3. 5. Partial sequence of cytochrome *c* subunit 1 gene with amino acid translation (*=change in amino acid in other species);
number after species name is the number of samples in each group; N=A, C, G, or T.



	Phe	Val	Ile	Ile	Phe	Phe	Ile	Val	Ile	Pro	Ile	Met	Ile	Gly	Gly	Phe	Gly	Asn	Trp	Leu	Ile
Saurida sp2 1 nws	T T C	G T T	A T A	A T T	T T C	T T T	A T A	G T A	A T A	C C A	A T C	A T G	A T C	G G G	G G G	T T T	G G A	A A C	T G G	C T A	A T T
Saurida sp2 2 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 3 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 4 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
S. undosquamis 1 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	. . .	. . A	. . T	. . C
S. undosquamis 2 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	. . .	. . A	. . T	. . C
S. undosquamis 3 nws	. . .	. . C	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	. . .	. . A	. . T	. . C
S. undosquamis 4 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	. . .	. . .	. . A	. . T	. . C
S. cf *argentea* 1 goc	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . T	. . A	. . C	. . .	. . .	. . A	. . C	. . .
S. cf *argentea* 2 goc	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . T	. . A	. . C	. . .	. . .	. . A	. . C	. . .
S. cf *argentea* 3 goc	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . T	. . A	. . C	. . .	. . .	. . A	. . C	. . .
S. cf *argentea* 4 goc	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . T	. . A	. . C	. . .	. . .	. . A	. . C	. . .
S. filamentosa 1 goc	. . .	. . G	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . C	. . A	. . C	. . .	. . .	. . A	. . .	. . C
S. filamentosa 2 goc	. . .	. . G	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . C	. . A	. . C	. . .	. . .	. . A	. . .	. . C
S. filamentosa 3 goc	. . .	. . G	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . N	. . A	. . C	. . .	. . .	. . A	. . .	. . C
S. longimanus 1 nws	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . A	. . T	. . C	. . C	. . C	. . .	. . .	. . A	. . T	. . .
S. longimanus 2 nws	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . A	. . T	. . C	. . C	. . C	. . .	. . .	. . A	. . T	. . C
S. longimanus 3 nws	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . A	. . T	. . C	. . C	. . C	. . .	. . .	. . A	. . T	. . C
S. longimanus 4 nws	. . .	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . T	. . A	. . T	. . C	. . C	. . C	. . .	. . .	. . A	. . T	. . C
			6660			6670			6680				6690			6700			6710		

	Pro	Leu	Ile	Ile	Gly	Ala	Pro	Asp	Met	Ala	Phe	Pro	Arg	Met	Asn	Asn	Met	Ser*	Phe	Leu	Leu
Saurida sp2 1 nws	C C C	C T A	A T A	A T C	G G T	G C C	C C T	G A C	A T G	G C A	T T T	C C T	C G C	A T G	A A C	A A C	A T G	A G C	T T C	T G G	C T T
Saurida sp2 2 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 3 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 4 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
S. undosquamis 1 nws	. . .	. . T	. . .	. . T	. . C	. . .	. . .	. . .	. . A	. . .	. . C	. . .	. . .	. . .	. . T	. . .	. . A	. . .	. . .	. . A	. . .
S. undosquamis 2 nws	. . .	. . T	. . .	. . T	. . C	. . .	. . .	. . .	. . A	. . .	. . C	. . .	. . .	. . .	. . T	. . .	. . A	. . .	. . .	. . A	. . .
S. undosquamis 3 nws	. . .	. . T	. . .	. . T	. . C	. . .	. . .	. . .	. . A	. . .	. . C	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . A	. . .
S. undosquamis 4 nws	. . .	. . T	. . .	. . T	. . C	. . .	. . .	. . .	. . A	. . .	. . C	. . .	. . .	. . .	. . T	. . .	. . A	. . .	. . .	. . A	. . .
S. cf *argentea* 1 goc	. . .	. . C	. . G	. . .	. . G	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . A	. . A	. . .	. . .	. . A	. . .	. . T	. . A	. . .
S. cf *argentea* 2 goc	. . .	. . C	. . G	. . .	. . G	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . A	. . A	. . .	. . .	. . A	. . .	. . T	. . A	. . .
S. cf *argentea* 3 goc	. . .	. . C	. . G	. . .	. . G	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . A	. . A	. . .	. . .	. . A	. . .	. . T	. . A	. . .
S. cf *argentea* 4 goc	. . .	. . C	. . G	. . .	. . G	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . A	. . A	. . .	. . .	. . A	. . .	. . T	. . A	. . .
S. filamentosa 1 goc	. . .	. . T	. . G	. . T	. . A	. . .	. . C	. . T	. . .	. . .	. . .	. . C	. . A	. . A	. . .	. . T	. . A	G . .	. . T	. . A	. . C
S. filamentosa 2 goc	. . .	. . T	. . G	. . T	. . A	. . .	. . C	. . T	. . .	. . .	. . .	. . C	. . A	. . A	. . .	. . T	. . A	G . .	. . T	. . A	. . C
S. filamentosa 3 goc	. . .	. . T	. . G	. . T	. . A	. . .	. . C	. . T	. . .	. . .	. . .	. . C	. . A	. . A	. . .	. . T	. . A	G . .	. . T	. . A	. . C
S. longimanus 1 nws	. . .	. . T	. . G	. . T	. . A	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . T	. . .	. . T	. . .	. . A	. . T	. . .	. . A	. . .
S. longimanus 2 nws	. . .	. . T	. . G	. . T	. . A	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . T	. . .	. . T	. . .	. . A	. . T	. . .	. . A	. . .
S. longimanus 3 nws	. . .	. . T	. . G	. . T	. . A	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . T	. . .	. . T	. . .	. . A	. . T	. . .	. . A	. . .
S. longimanus 4 nws	. . .	. . T	. . G	. . T	. . A	. . .	. . C	. . .	. . .	. . .	. . .	. . C	. . T	. . .	. . T	. . .	. . A	. . T	. . .	. . A	. . .
			6720			6730			6740			6750			6760			6770			


Leu Pro Pro Ser Phe Leu Leu Leu Leu Ala Ser Ser Gly Val Glu Ala Gly Ala Gly Thr Gly
C T T C C C C C C T C C T C C C C T C C C C T C T G G G G T A G A A G C C G G A G C T G G G A C G T
Saurida sp2 1 nws
Saurida sp2 2 nws
Saurida sp2 3 nws
Saurida sp2 4 nws
S. undosquamis 1 nws
S. undosquamis 2 nws
S. undosquamis 3 nws
S. undosquamis 4 nws
S. cf argentea 1 goc
S. cf argentea 2 goc
S. cf argentea 3 goc
S. cf argentea 4 goc
S. filamentosa 1 goc
S. filamentosa 2 goc
S. filamentosa 3 goc
S. longimanus 1 nws
S. longimanus 2 nws
S. longimanus 3 nws
S. longimanus 4 nws
6780
6790
6800
6810
6820
6830
6840
stop Thr Val Tyr Pro Pro Leu Ala Gly Asn Leu Ala His Ala Gly Ala Ser Val Asp Leu* Thr
T G A A C A G T T T A C C C G C C C C T G G C A T A C T C T T A A C C
Saurida sp2 1 nws
Saurida sp2 2 nws
Saurida sp2 3 nws
Saurida sp2 4 nws
S. undosquamis 1 nws
S. undosquamis 2 nws
S. undosquamis 3 nws
S. undosquamis 4 nws
S. cf argentea 1 goc
S. cf argentea 2 goc
S. cf argentea 3 goc
S. cf argentea 4 goc
S. filamentosa 1 goc
S. filamentosa 2 goc
S. filamentosa 3 goc
S. longimanus 1 nws
S. longimanus 2 nws
S. longimanus 3 nws
S. longimanus 4 nws
6850
6860
6870
6880
6890
6900

	Ile	Phe	Ser	Leu	His	Leu	Ala	Gly	Ile	Ser	Ser	Ile	Stop*	Gly	Ala	Ile	Asn	Phe	Ile	Thr	Thr
Saurida sp2 1 nws	A T C	T T C	T C T	C T A	C A T	T T G	G C A	G G G	A T T	T C T	T C C	A T C	T A A	G G G	G C C	A T T	A A T	T T T	A T T	A C T	A C A
Saurida sp2 2 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 3 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 4 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
S. undosquamis 1 nws	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . C	. . .	. . .	. . G	. . .	. . T	. . .	. . C	. . .	. . .	. . .	. . .
S. undosquamis 2 nws	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . C	. . .	. . .	. . G	. . .	. . T	. . .	. . C	. . .	. . .	. . .	. . .
S. undosquamis 3 nws	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . C	. . .	. . .	. . G	. . .	. . T	. . .	. . C	. . .	. . .	. . .	. . .
S. undosquamis 4 nws	. . .	. . .	. . .	. . .	. . .	. . A	. . .	. . .	. . .	. . C	. . .	. . .	. . G	. . .	. . T	. . .	. . C	. . .	. . .	. . .	. . .
S. cf *argentea* 1 goc	. . T	. . T	. . A	. . T	. . C	C . T	. . .	. . .	. . .	. . C	. . .	. . .	C T .	. . .	. . .	. . .	. . C	. . .	. . .	. . C	. . .
S. cf *argentea* 2 goc	. . T	. . T	. . A	. . T	. . C	C . T	. . .	. . .	. . .	. . C	. . .	. . .	C T .	. . .	. . .	. . .	. . C	. . .	. . .	. . C	. . .
S. cf *argentea* 3 goc	. . T	. . T	. . A	. . T	. . C	C . T	. . .	. . .	. . .	. . C	. . .	. . .	C T .	. . .	. . .	. . .	. . C	. . .	. . .	. . C	. . .
S. cf *argentea* 4 goc	. . T	. . T	. . A	. . T	. . C	C . T	. . .	. . .	. . .	. . C	. . .	. . .	C T .	. . .	. . .	. . .	. . C	. . .	. . .	. . C	. . .
S. filamentosa 1 goc	. . T	. . T	. . .	. . C	. . C	C . .	. . T	. . A	. . C	. . C	. . T	. . .	C T G	. . .	. . .	. . .	. . C	. . .	. . .	. . C	. . .
S. filamentosa 2 goc	. . T	. . T	. . .	. . C	. . C	C . .	. . T	. . A	. . C	. . C	. . T	. . .	C T G	. . .	. . .	. . .	. . C	. . .	. . .	. . C	. . .
S. filamentosa 3 goc	. . T	. . T	. . .	. . C	. . C	C . .	. . T	. . A	. . C	. . C	. . T	. . .	C T G	. . .	. . .	. . .	. . C	. . .	. . .	. . C	. . .
S. longimanus 1 nws	. . .	. . T	. . C	. . C	. . C	C . A	. . G	. . A	. . .	. . C	. . .	. . T	C T T	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .
S. longimanus 2 nws	. . .	. . T	. . C	. . C	. . C	C . A	. . G	. . A	. . .	. . C	. . .	. . T	C T T	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .
S. longimanus 3 nws	. . .	. . T	. . C	. . C	. . C	C . A	. . G	. . A	. . .	. . C	. . .	. . T	C T T	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .
S. longimanus 4 nws	. . .	. . T	. . C	. . C	. . C	C . A	. . G	. . A	. . .	. . C	. . .	. . T	C T T	. . A	. . .	. . .	. . .	. . .	. . .	. . .	. . .
		6910			6920			6930			6940				6950				6960		

	Ile	Ile	Thr*	Leu*	Lys	Pro	Pro	Ala	Ile	Ser	Glu	Tyr	Gln	Thr	Pro	Leu	Phe	Phe	Stop	Thr*	Phe
Saurida sp2 1 nws	A T T	A T C	A C T	T T A	A A A	C C C	C C T	G C C	A T C	T C A	C A A	T A C	C A A	A C C	C C C	T T G	T T T	T T T	T G A	A C A	T T C
Saurida sp2 2 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 3 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Saurida sp2 4 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
S. undosquamis 1 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . C	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	C . A	. . .	. . .	. . .	. . .	. . .
S. undosquamis 2 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . C	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	C . A	. . .	. . .	. . .	. . .	. . .
S. undosquamis 3 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . C	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	C . A	. . .	. . .	. . .	. . .	. . .
S. undosquamis 4 nws	. . .	. . .	. . .	. . .	. . .	. . .	. . C	. . .	. . .	. . .	. . .	. . T	. . .	. . .	. . .	C . A	. . .	. . .	. . .	. . .	. . .
S. cf *argentea* 1 goc	. . C	. . T	. A .	A . G	. . .	. . T	. . C	. . T	. . T	. . .	. . .	. . T	. . .	. . .	. . A	C . A	. . .	G . .	. . .	G . G	G . .
S. cf *argentea* 2 goc	. . C	. . T	. A .	A . G	. . .	. . T	. . C	. . T	. . T	. . .	. . .	. . T	. . .	. . .	. . A	C . A	. . .	G . .	. . .	G . G	G . .
S. cf *argentea* 3 goc	. . C	. . T	. A .	A . G	. . .	. . T	. . C	. . T	. . T	. . .	. . .	. . T	. . .	. . .	. . A	C . A	. . .	G . .	. . .	G . G	G . .
S. cf *argentea* 4 goc	. . .	. . T	. A .	A . G	. . .	. . T	. . C	. . T	. . T	. . .	. . .	. . T	. . .	. . .	. . A	C . A	. . .	G . .	. . .	G . G	G . .
S. filamentosa 1 goc	. . .	. . T	. A C	A . G	. . .	. . .	. . C	. . T	. . .	. . .	. . .	. . T	. . .	. . .	. . G	C . A	. . .	G . .	. . G	G . .	G . .
S. filamentosa 2 goc	. . .	. . T	. A C	A . G	. . .	. . .	. . C	. . T	. . .	. . .	. . .	. . T	. . .	. . .	. . G	C . A	. . .	G . .	. . G	G . .	G . .
S. filamentosa 3 goc	. . .	. . T	. A C	A . G	. . .	. . .	. . C	. . T	. . .	. . .	. . .	. . T	. . .	. . .	. . G	C . A	. . .	G . .	. . G	G . .	G . .
S. longimanus 1 nws	. . .	. . T	. A .	A . G	. . G	. . .	. . .	. . T	. . T	. . .	. . .	. . .	. . .	. . .	. . .	C . C	. . .	G . .	. . .	. . G	G . .
S. longimanus 2 nws	. . .	. . T	. A .	A . G	. . G	. . .	. . .	. . T	. . T	. . .	. . .	. . .	. . .	. . .	. . .	C . C	. . .	G . .	. . .	. . G	G . .
S. longimanus 3 nws	. . .	. . T	. A .	A . G	. . G	. . .	. . .	. . T	. . T	. . .	. . .	. . .	. . .	. . .	. . .	C . C	. . .	G . .	. . .	. . G	G . .
S. longimanus 4 nws	. . .	. . T	. A .	A . G	. . G	. . .	. . .	. . T	. . T	. . .	. . .	. . .	. . .	. . .	. . .	C . C	. . .	G . .	. . .	. . G	G . .
	6970			6980				6990			7000				7010			7020			7030

	Leu			Ile			Thr			Ala			Val			Leu			Leu			Leu			Leu			Ser			Leu			Pro			Val			Leu		
Saurida sp2 1 nws	C	T	A	A	T	T	A	C	C	G	C	C	G	T	C	C	T	T	C	T	T	C	T	G	C	T	C	T	C	C	C	T	C	C	T	G	T	T	C	T	C
Saurida sp2 2 nws	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Saurida sp2 3 nws	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
Saurida sp2 4 nws	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. undosquamis 1 nws	.	.	C	.	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T
S. undosquamis 2 nws	.	.	C	.	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T
S. undosquamis 3 nws	.	.	C	.	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T
S. undosquamis 4 nws	.	.	C	.	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T
S. cf *argentea* 1 goc	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	T
S. cf *argentea* 2 goc	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	N
S. cf *argentea* 3 goc	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	T
S. cf *argentea* 4 goc	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	T
S. filamentosa 1 goc	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.	A	.	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	C	.	.	.	.	.	T
S. filamentosa 2 goc	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.	A	.	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	C	.	.	.	.	.	T
S. filamentosa 3 goc	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.	A	.	.	.	.	.	C	.	.	T	.	.	T	.	.	.	.	.	C	.	.	.	.	.	T
S. longimanus 1 nws	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.	T	.	.	T	.	.	T	.	.	C	.	.	C	.	.	T
S. longimanus 2 nws	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.	T	.	.	T	.	.	T	.	.	C	.	.	C	.	.	T
S. longimanus 3 nws	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	.	.	T	.	.	T	.	.	C	.	.	C	.	.	T
S. longimanus 4 nws	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	T	.	.	T	.	.	T	.	.	C	.	.	C	.	.	T
									7040										7050									7060									7070			7073		

Table 3. 13. Absolute distances (below diagonal) that represents base substitution (transversion=above, transition =below) and mean distances that represents proportion of divergence in cytochrome oxidase *c* subunit 1 gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 *Saurida* sp2 1 nws	-	0.002	0.007	0	0.101	0.099	0.099	0.099	0.17	0.167	0.17	0.168	0.181	0.181	0.178	0.174	0.176	0.174	0.176
2 *Saurida* sp2 2 nws	0 1	-	0.005	0.002	0.099	0.097	0.097	0.097	0.172	0.168	0.172	0.17	0.183	0.183	0.179	0.172	0.174	0.172	0.174
3 *Saurida* sp2 3 nws	0 4	0 3	-	0.007	0.099	0.097	0.097	0.097	0.176	0.172	0.176	0.174	0.179	0.179	0.176	0.167	0.168	0.167	0.168
4 *Saurida* sp2 4 nws	0 0	0 1	0 4	-	0.101	0.099	0.099	0.099	0.17	0.167	0.17	0.168	0.181	0.181	0.178	0.174	0.176	0.174	0.176
5 *S. undosquamis* 1nws	8 47	8 46	7 47	8 47	-	0.002	0.005	0.002	0.154	0.152	0.154	0.152	0.165	0.163	0.159	0.168	0.167	0.168	0.167
6 *S. undosquamis* 2 nws	8 46	8 45	7 46	8 46	0 1	-	0.004	0	0.152	0.15	0.152	0.15	0.163	0.161	0.158	0.167	0.165	0.167	0.165
7 *S. undosquamis* 3 nws	8 46	8 45	7 46	8 46	0 3	0 2	-	0.004	0.15	0.148	0.15	0.148	0.161	0.159	0.156	0.168	0.167	0.168	0.167
8 *S. undosquamis* 4 nws	8 46	8 45	7 46	8 46	0 1	0 0	0 2	-	0.152	0.15	0.152	0.15	0.163	0.161	0.158	0.167	0.165	0.167	0.165
9 *S.* cf *argentea* 1 goc	23 70	23 71	22 74	23 70	19 65	19 64	19 63	19 64	-	0.002	0	0.002	0.115	0.114	0.11	0.143	0.145	0.143	0.141
10 *S.* cf *argentea* 2 goc	23 68	23 69	22 72	23 68	19 64	19 63	19 62	19 63	0 1	-	0.002	0.004	0.114	0.112	0.108	0.145	0.147	0.145	0.143
11 *S.* cf *argentea* 3 goc	23 70	23 71	22 74	23 70	19 65	19 64	19 63	19 64	0 0	0 1	-	0.002	0.115	0.114	0.11	0.143	0.145	0.143	0.141
12 *S.* cf *argentea* 4 goc	23 69	23 70	22 73	23 69	19 64	19 63	19 62	19 63	0 1	0 2	0 1	-	0.114	0.112	0.108	0.141	0.143	0.141	0.139
13 *S. filamentosa* 1goc	26 73	26 74	25 73	26 73	24 66	24 65	24 64	24 65	11 52	11 51	11 52	11 51	-	0.002	0.002	0.152	0.15	0.152	0.154
14 *S.filamentosa* 2 goc	26 73	26 74	25 73	26 73	24 65	24 64	24 63	24 64	11 51	11 50	11 51	11 50	0 1	-	0	0.152	0.15	0.152	0.154
15 *S.filamentosa* 3 goc	25 72	25 73	24 72	25 72	24 63	24 62	24 61	24 62	11 49	11 48	11 49	11 48	0 1	0 0	-	0.15	0.148	0.15	0.152
16 *S. longimanus* 1 nws	24 71	24 70	24 67	24 71	24 68	24 67	24 68	24 67	17 61	17 62	17 61	17 60	20 63	20 63	20 62	-	0.002	0.004	0.005
17 *S. longimanus* 2 nws	24 72	24 71	24 68	24 72	24 67	24 66	24 67	24 66	17 62	17 63	17 62	17 61	20 62	20 62	20 61	0 1	-	0.002	0.004
18 *S. longimanus* 3 nws	24 71	24 70	24 67	24 71	24 68	24 67	24 68	24 67	17 61	17 62	17 61	17 60	20 63	20 63	20 62	0 2	0 1	-	0.002
19 *S. longimanus* 4 nws	24 72	24 71	24 68	24 72	24 67	24 66	24 67	24 66	17 60	17 61	17 60	17 59	20 64	20 64	20 63	0 3	0 2	0 1	-

National Centre for Biotechnology Information (NCBI) database. The *O. mykiss* sequence was subsequently used as an outgroup taxon for phylogenetic analysis.

Uncorrected pairwise distances between the the species are presented in Table 3.13. Intraspecific distances tended to be small and ranged from 0-0.2% in *S. filamentosa* to 0-0.7% in *Saurida* species 2. Interspecific distances ranged from 9.7-10.1% for pairwise comparisons between *Saurida* species 2 and *S. undosquamis* to over 18% between *Saurida* species 2 and *S. filamentosa.*

The overall nucleotide composition for first, second and third codon positions within the COI fragment is given in Figure 3.6. Thymine and cytosine occur more frequently than the purine bases. The pyrimidine bases and adenine occur at similar frequencies at third codon positions whereas thymine tended to have the highest frequency in second codon positions.

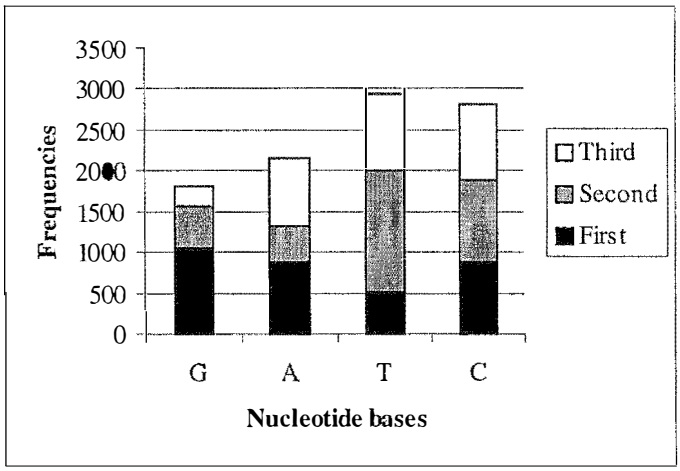


Figure 3. 6. Nucleotide base composition and its codon position within cytochrome oxidase *c* subunit 1 gene.

Frequencies of transition and transversion nucleotide base substitution and percent of divergence resulting from pairwise comparison between taxa is depicted in Figure 3.7. Some base substitutions in the first and third codon positions were responsible in the replacement of four amino acids. Two amino acid replacements were brought about by nucleotide base substitution at second codon positions. Other base substitutions (8, 1, 125 at first, second, and third codon positions, respectively) were conservative, not resulting in amino acid replacements.

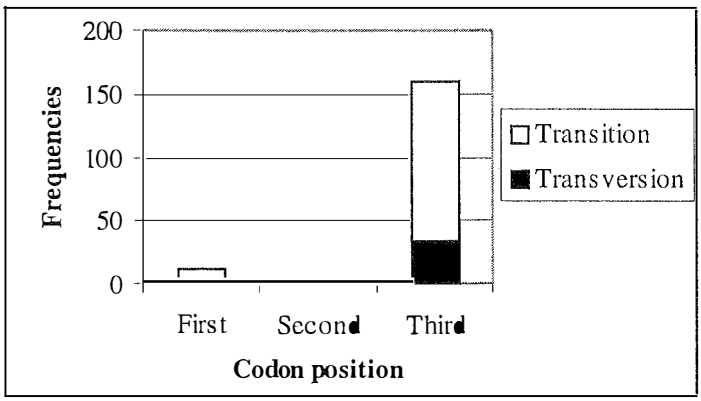


Figure 3. 7. Frequencies of transversion and transition and their codon positions whithin cytochrome oxidase *c* subunit 1 gene.

A comparison of pairwise divergence values yielded a transition (Ti):transversion (Tv) ratio of 3.2:1. A similar ratio was also obtained by comparing the slope of transition and the slope of transversion. The maximum transition/transversion ratios were obtained from comparison of *Saurida* sp2. and *S. undosquamis* (6.7:1). This result indicated a substitutional bias in the ratio of transition/transversion in mitochondrial genome of animals since in the comparison of closely related taxa, it may exceed 10:1 (Brown, 1985).

Transition and transversion substitutions were analysed using three weighting schemes during parsimony analysis. These were equal weighting, increased weighting of transversions by a factor of three over transitions and analysis of transversions only.

The result of unweighted maximum parsimony analysis of the cytochrome oxidase subunit I gene (20 trees, 306 steps) is presented in Figure 3.8a. The five species of saurids are clearly separated with strong bootstrap support and occur in two distinct assemblages. *Saurida* sp2 and *S. undosquamis* have been grouped in one clade as sister taxa. *Saurida* cf *argentea* and *S. Filamentosa* are grouped as sister taxa in the second assemblage with *S. longimanus* forming a separate clade. However, the latter assemblage has reduced bootstrap support.

A 3:1 Tv:Ti weighted parsimony analysis (40 trees, 467 steps) resulted in a largely congruent topology with similar levels of bootstrap support, with the exception of the *S.*

longimanus clade which was placed basal to the *S.*cf *argentea* and *S. Filamentosa* assemblage (Figure 3.8b).

Weighting transversions alone (4 trees, 79 steps) successfully separated the five species however, bootstrap support for the two assemblage was reduced (Figure 3.8c).

The DNADIST application of the PHYLIP version 3.5c package (Felsenstein, 1993), was used to correct pairwise distance for multiple substitutions using the Kimura two-parameter model (Kimura, 1980) assuming a Ti:Tv ratio of 2:1. The results are presented in Figure 3.9 where a strong linear relationship is evident for both Ti and Tv frequencies relative to corrected distance.

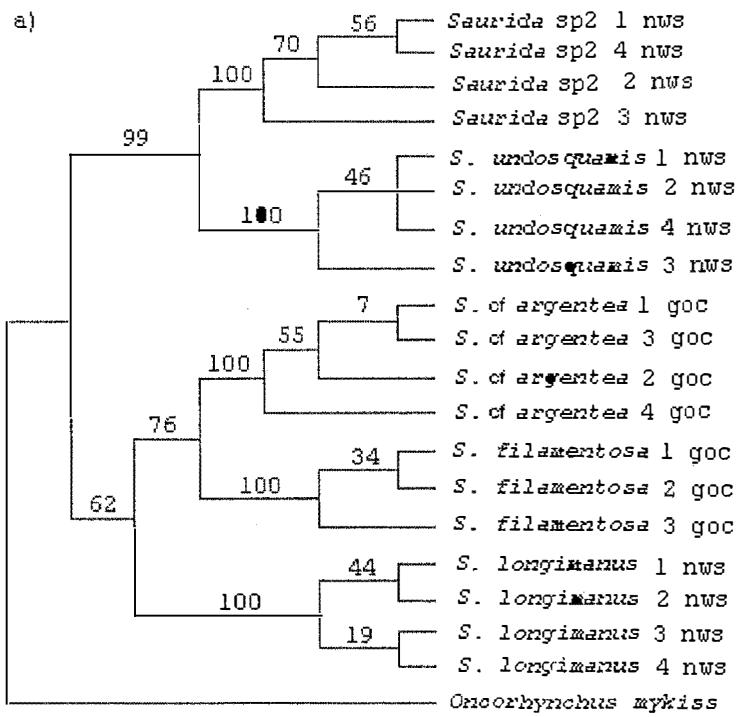
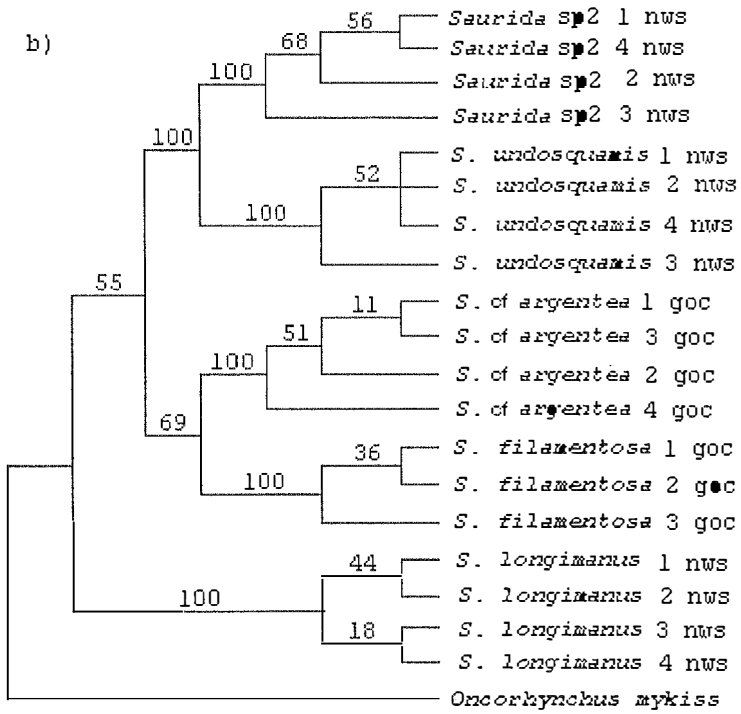


Figure 3. 8. Bootstrap 50% majority-rule consensus tree on cytochrome oxidase *c* subunit 1 gene; after species name are the location and sample's numbering; a) unweighted analysis, b) weighted to transversion=3 and transition=1, c) weighted to transversion alone.


b)
56
68
100
100
52
100
55
11
51
100
69
36
100
44
100
18
Saurida sp2 1 nws
Saurida sp2 4 nws
Saurida sp2 2 nws
Saurida sp2 3 nws
S. undosquamis 1 nws
S. undosquamis 2 nws
S. undosquamis 4 nws
S. undosquamis 3 nws
S. cf argentea 1 goc
S. cf argentea 3 goc
S. cf argentea 2 goc
S. cf argentea 4 goc
S. filamentosa 1 goc
S. filamentosa 2 goc
S. filamentosa 3 goc
S. longimanus 1 nws
S. longimanus 2 nws
S. longimanus 3 nws
S. longimanus 4 nws
Oncorhynchus mykiss

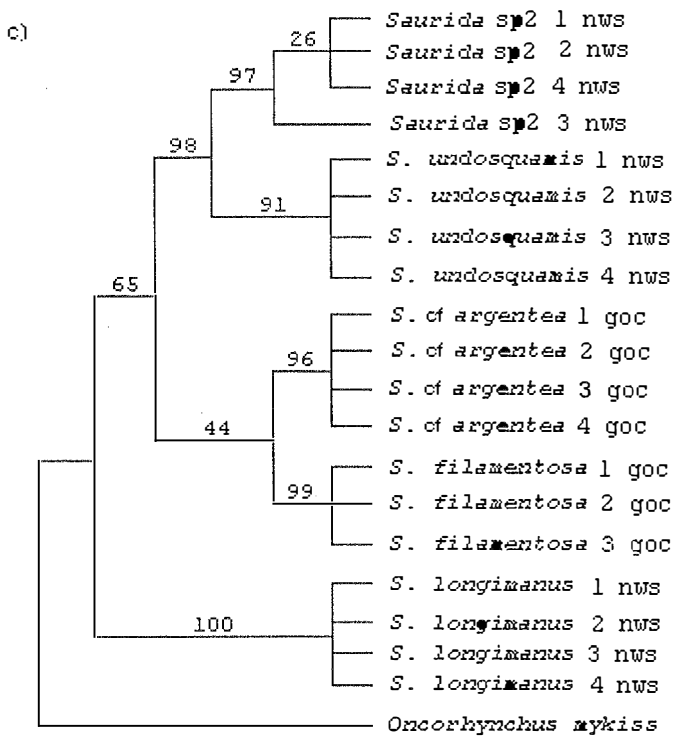

c)
26
97
98
91
65
96
44
99
100
Saurida sp2 1 nws
Saurida sp2 2 nws
Saurida sp2 4 nws
Saurida sp2 3 nws
S. undosquamis 1 nws
S. undosquamis 2 nws
S. undosquamis 3 nws
S. undosquamis 4 nws
S. cf argentea 1 goc
S. cf argentea 2 goc
S. cf argentea 3 goc
S. cf argentea 4 goc
S. filamentosa 1 goc
S. filamentosa 2 goc
S. filamentosa 3 goc
S. longimanus 1 nws
S. longimanus 2 nws
S. longimanus 3 nws
S. longimanus 4 nws
Oncorhynchus mykiss

Figure 3.9 Frequencies of Ti and Tv substitutions relative to corrected distance (Kimura 2-parameter) for the COI gene.

3. 2b. Comparison of mitochondrial cytochrome b gene.

The same samples used to determine the sequence identity of the COI gene were used for the analysis of cytochrome b, with the addition of *Saurida* sp2 samples from the Gulf of Carpentaria (GOC) and Queensland (QLD) and *S. filamentosa* samples from QLD to examine geographical variation.

The sequenced cytochrome b fragment was 308 bp in length coding for 102 amino acids (Figure 3.10). A BLAST search of the NCBI database found the cytochrome b gene in *Hemichromis bimaculatis* to be the closest match and it was subsequently used as a reference sequence and outgroup taxon.

Pairwise distances between taxa are presented in Table 3.14. Intraspecific distances were low and ranged from 0-0.16% in *S. filamentosa* to 0-4.2% in *Saurida* sp2. Interspecific distances were greater, the highest ranging from 15.6-16.9% for comparisons between *S. longimanus* (NSW) and *S.* cf *argentea* (GOC). The remaining pairwise comparisons ranged from 10.4-16.2%.

The nucleotide base composition for each codon position within the cytochrome b gene fragment is given in Figure 3.11. The four nucleotides occurred in almost equal

Figure 3. 10. Partial sequence of mitochondrial cytochrome b gene with amino acid translation (*=change in amino acid in other species); nws=North West Shelf, GOC=Gulf of Carpentaria, Qld=Queensland.

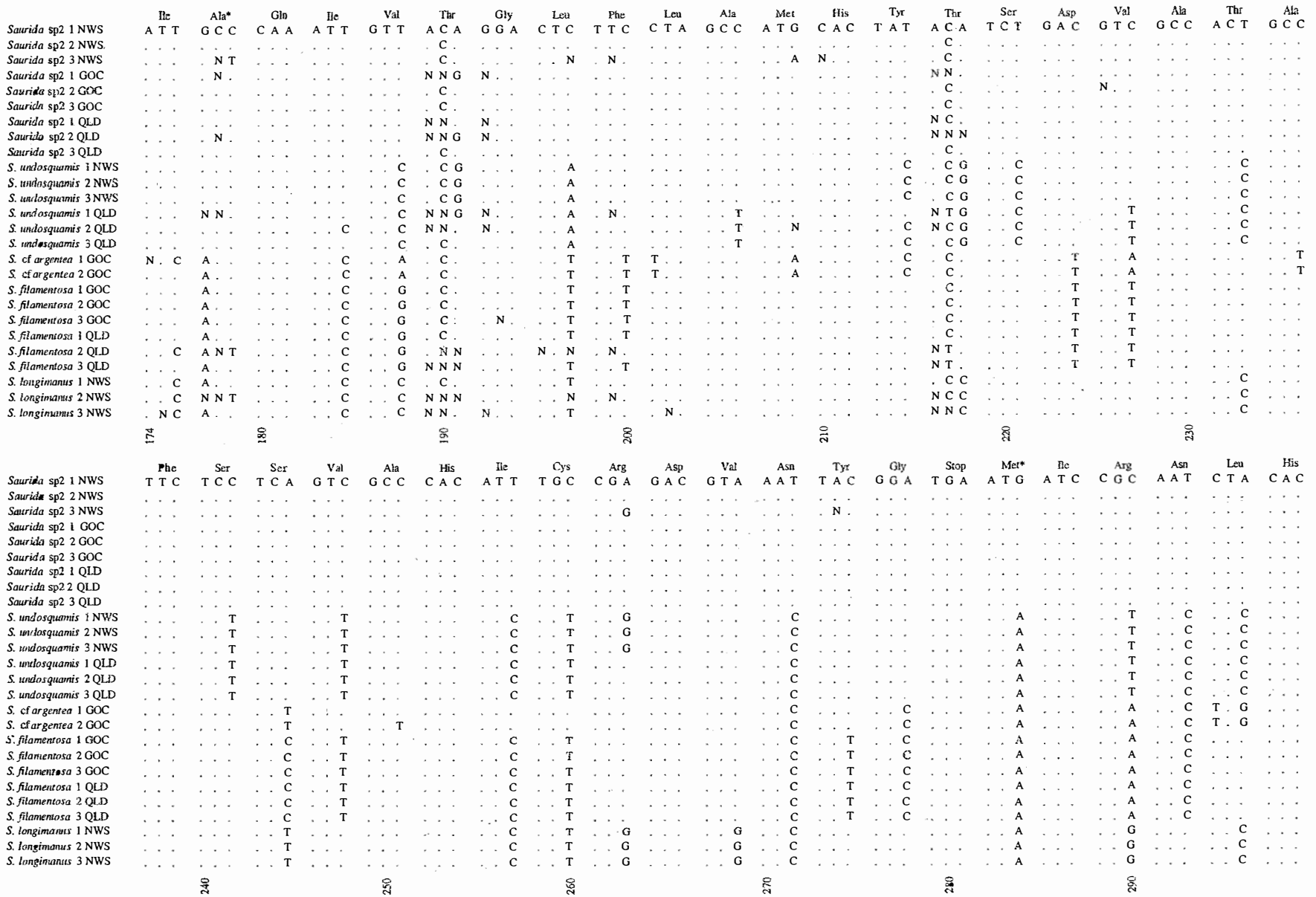


Figure 3. 10 (*Continued*)

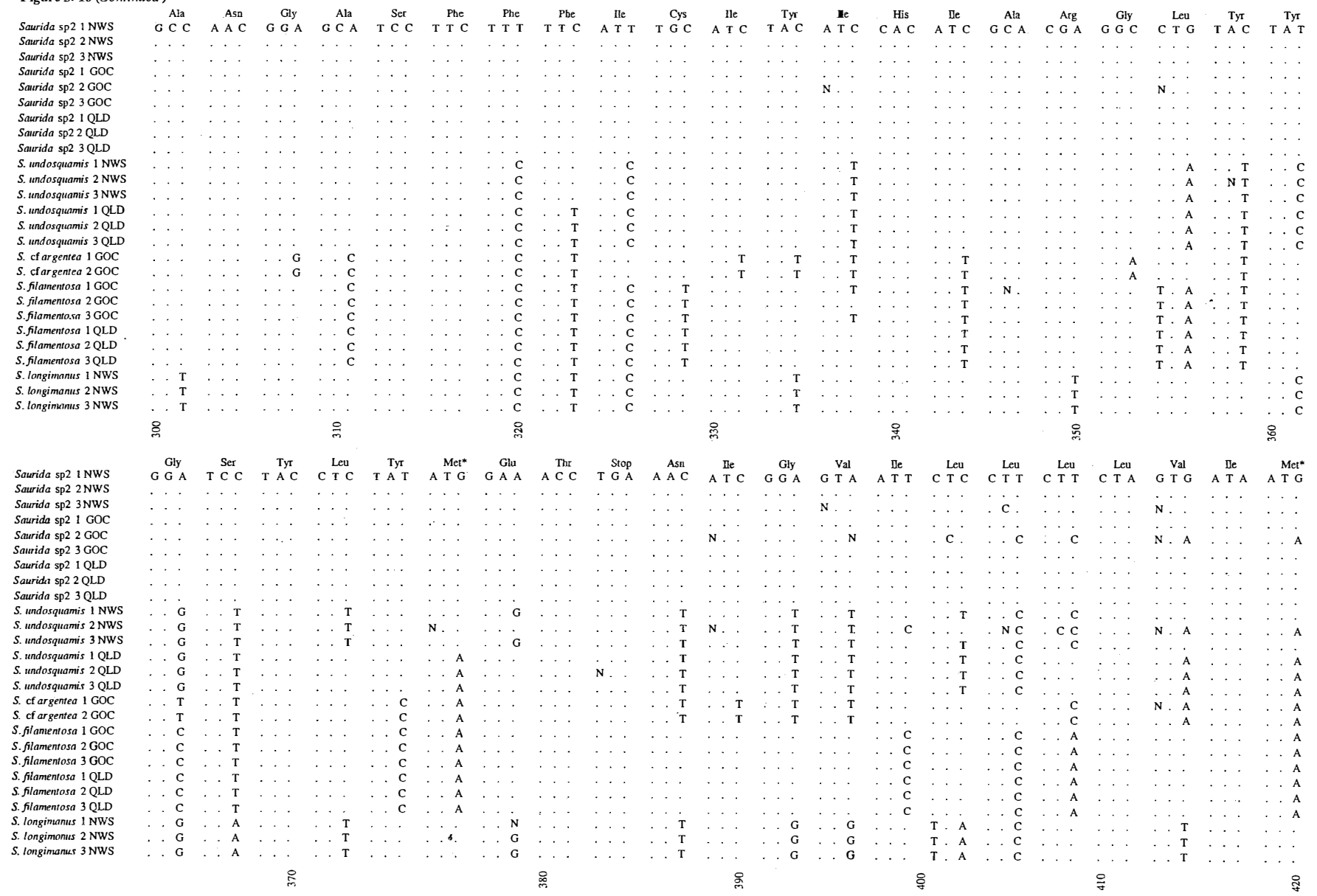


Figure 3. 10 (*Continued*)

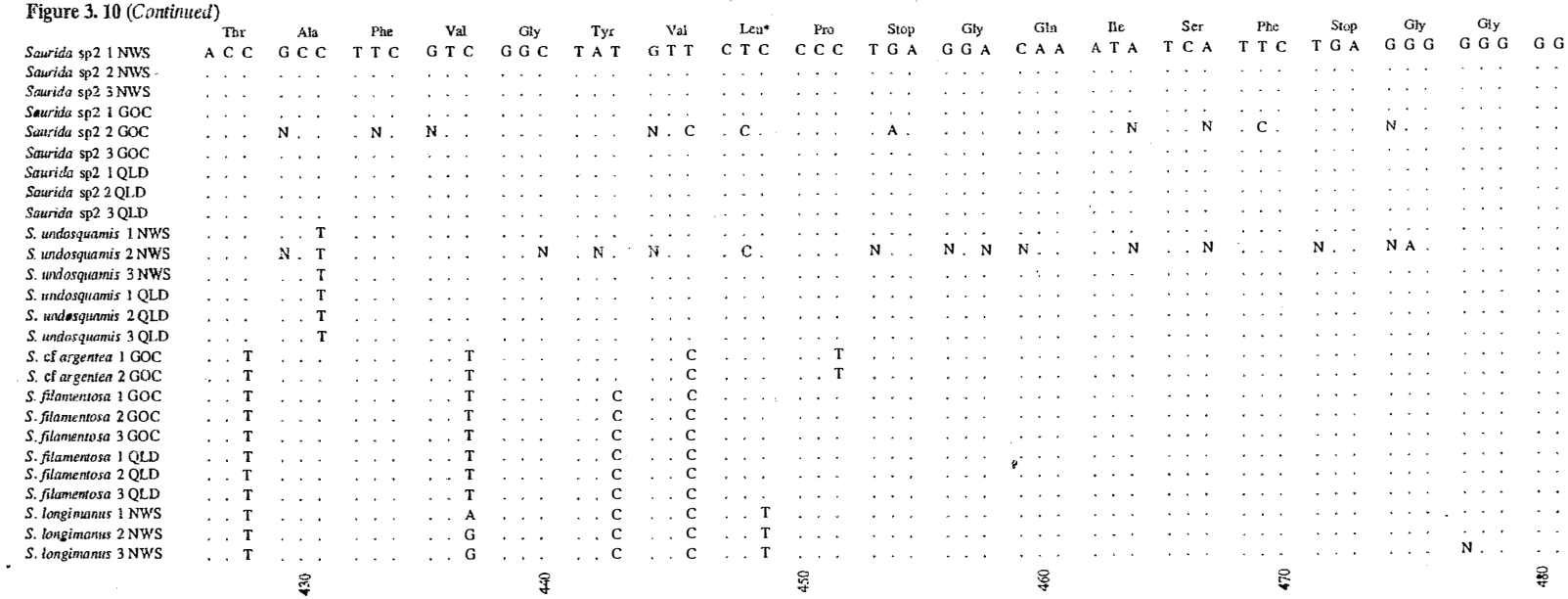


Table 3.15. Absolute distances (below diagonal), represent base substitution (transversion=above, transition=below), and mean distances (above diagonal), represent proportion of divergence (uncorrected) in cytochrome b gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1 *Saurida* sp2 1 nws	-	0	0.013	0.003	0.029	0	0	0.003	0	0.11	0.123	0.11	0.117	0.117	0.114	0.146	0.146	0.127	0.123	0.127	0.123	0.127	0.127	0.123	0.123	0.127
2 *Saurida* sp2 2 nws	0 0	-	0.013	0.003	0.029	0	0	0.003	0	0.11	0.123	0.11	0.117	0.117	0.114	0.146	0.146	0.127	0.123	0.127	0.123	0.127	0.127	0.123	0.123	0.127
3 *Saurida* sp2 3 nws	0 4	0 4	-	0.016	0.042	0.013	0.013	0.016	0.013	0.114	0.123	0.114	0.127	0.123	0.123	0.149	0.149	0.136	0.133	0.136	0.133	0.133	0.136	0.127	0.123	0.13
4 *Saurida* sp2 1goc	0 1	0 1	0 5	-	0.032	0.003	0.003	0	0.003	0.107	0.12	0.107	0.11	0.12	0.117	0.149	0.149	0.13	0.127	0.13	0.127	0.123	0.123	0.127	0.123	0.13
5 *Saurida* sp2 2goc	0 9	0 9	0 13	0 10	-	0.029	0.029	0.032	0.029	0.123	0.117	0.123	0.123	0.123	0.12	0.146	0.146	0.13	0.127	0.13	0.127	0.13	0.13	0.133	0.133	0.136
6 *Saurida* sp2 3 goc	0 0	0 0	0 4	0 1	0 9	-	0	0.003	0	0.11	0.123	0.11	0.117	0.117	0.114	0.146	0.146	0.127	0.123	0.127	0.123	0.127	0.127	0.123	0.123	0.127
7 *Saurida* sp2 1qld	0 0	0 0	0 4	0 1	0 9	0 0	-	0.003	0	0.11	0.123	0.11	0.117	0.117	0.114	0.146	0.146	0.127	0.123	0.127	0.123	0.127	0.127	0.123	0.123	0.127
8 *Saurida* sp2 2 qld	0 1	0 1	0 5	0 0	0 10	0 1	0 1	-	0.003	0.104	0.117	0.104	0.107	0.117	0.114	0.149	0.149	0.13	0.127	0.13	0.127	0.123	0.123	0.123	0.12	0.127
9 *Saurida* sp2 3 qld	0 0	0 0	0 4	0 1	0 9	0 0	0 0	0 1	-	0.11	0.123	0.11	0.117	0.117	0.114	0.146	0.146	0.127	0.123	0.127	0.123	0.127	0.127	0.123	0.123	0.127
10 *S. undosquamis* 1 nws	4 30	4 30	3 32	4 29	3 35	4 30	4 30	4 28	4 30	-	0.026	0	0.039	0.039	0.036	0.162	0.162	0.136	0.14	0.136	0.14	0.14	0.14	0.11	0.104	0.11
11 *S. undosquamis* 2 nws	4 34	4 34	3 35	4 33	3 33	4 34	4 34	4 32	4 34	0 8	-	0.026	0.045	0.045	0.042	0.162	0.162	0.136	0.14	0.136	0.14	0.14	0.14	0.127	0.123	0.13
12 *S. undosquamis* 3 nws	4 30	4 30	3 32	4 29	3 35	4 30	4 30	4 28	4 30	0 0	0 8	-	0.039	0.039	0.036	0.162	0.162	0.136	0.14	0.136	0.14	0.14	0.14	0.11	0.104	0.11
13 *S. undosquamis* 1 qld	4 32	4 32	3 36	4 30	3 35	4 32	4 32	4 29	4 32	0 12	0 14	0 12	-	0.013	0.01	0.149	0.149	0.117	0.12	0.117	0.12	0.114	0.114	0.12	0.12	0.12
14 *S. undosquamis* 2 qld	4 32	4 32	3 35	4 33	3 35	4 32	4 32	4 32	4 32	0 12	0 14	0 12	0 4	-	0.003	0.136	0.136	0.114	0.117	0.114	0.117	0.12	0.12	0.117	0.117	0.12
15 *S. undosquamis* 3 qld	4 31	4 31	3 35	4 32	3 34	4 31	4 31	4 31	4 31	0 11	0 13	0 11	0 3	0 1	-	0.143	0.143	0.117	0.12	0.117	0.12	0.123	0.123	0.12	0.12	0.123
16 *S. cfr argentea* 1 goc	10 35	10 35	10 36	10 36	9 36	10 35	10 35	10 36	10 35	10 40	10 40	10 40	10 36	10 32	10 34	-	0.006	0.107	0.11	0.107	0.11	0.117	0.114	0.156	0.162	0.159
17 *S. cf argentea* 2 goc	10 35	10 35	10 36	10 36	9 36	10 35	10 35	10 36	10 35	10 40	10 40	10 40	10 36	10 32	10 34	0 2	-	0.107	0.11	0.107	0.11	0.123	0.114	0.162	0.169	0.166
18 *S. filamentosa* 1 goc	7 32	7 32	7 35	7 33	7 33	7 32	7 32	7 31	7 32	11 31	11 31	11 31	11 25	11 24	11 25	5 28	5 28	-	0.003	0	0.003	0.016	0.006	0.14	0.146	0.143
19 *S. filamentosa* 2 goc	7 31	7 31	7 34	7 32	7 32	7 31	7 31	7 32	7 31	11 32	11 32	11 32	11 26	11 25	11 26	5 29	5 29	0 1	-	0.003	0	0.013	0.003	0.136	0.143	0.14
20 *S. filamentosa* 3 goc	7 32	7 32	7 35	7 33	7 33	7 32	7 32	7 33	7 32	11 31	11 31	11 31	11 25	11 24	11 25	5 28	5 28	0 0	0 1	-	0.003	0.016	0.006	0.14	0.146	0.143
21 *S. filamentosa* 1 qld	7 31	7 31	7 34	7 32	7 32	7 31	7 31	7 32	7 31	11 32	11 32	11 32	11 26	11 25	11 26	5 29	5 29	0 1	0 0	0 1	-	0.013	0.003	0.136	0.143	0.14
22 *S. filamentosa* 2 qld	7 32	7 32	7 34	7 31	7 33	7 32	7 32	7 31	7 32	10 33	10 33	10 33	10 25	10 27	10 28	5 31	5 33	0 5	0 4	0 5	0 4	-	0.01	0.136	0.136	0.136
23 *S. filamentosa* 3 qld	7 32	7 32	7 35	7 31	7 33	7 32	7 32	7 31	7 32	11 32	11 32	11 32	11 24	11 26	11 27	5 30	5 30	0 2	0 1	0 2	0 1	0 3	-	0.14	0.146	0.14
24 *S. longimanus* 1 nws	9 29	9 29	9 30	9 30	9 33	9 29	9 29	8 30	9 29	11 23	11 28	11 23	11 26	11 25	11 26	15 33	15 35	12 31	12 30	12 31	12 30	12 30	12 31	-	0.006	0.003
25 *S. longimanus* 2 nws	9 29	9 29	9 29	9 29	9 32	9 29	9 29	8 29	9 29	10 22	10 28	10 22	10 27	10 26	10 27	15 35	15 37	12 33	12 32	12 33	12 32	12 30	12 33	0 2	-	0.003
26 *S. longimanus* 3 nws	9 30	9 30	9 31	9 31	9 33	9 30	9 30	8 31	9 30	11 23	11 29	11 23	11 26	11 26	11 27	15 34	15 36	12 32	12 31	12 32	12 31	12 30	12 31	0 1	0 1	-

proportion in first codon positions. Thymine occurred most frequently in second codon positions whereas guanine occurred least frequently in third positions.

The frequencies of Ti and Tv substitutions relative to codon positions are given in Figure 3.12. Overall there were 10 amino acid replacements. A Ti:Tv ratio of 3:1 was determined from the pairwise distance matrix.

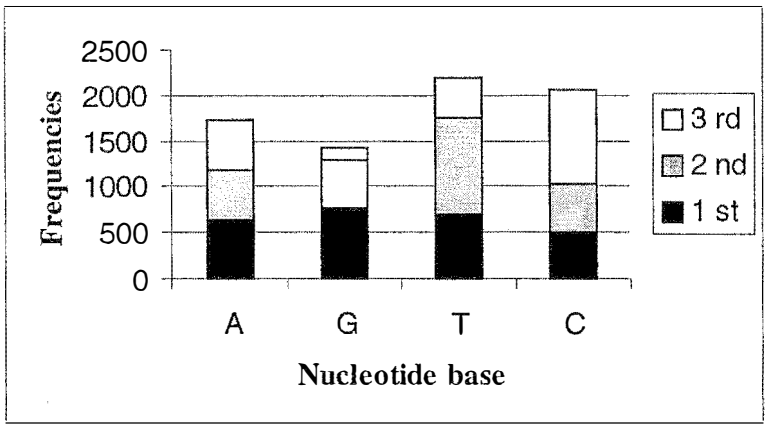


Figure 3. 11. Nucleotide base composition and its codon position within cytochrome *b* gene.

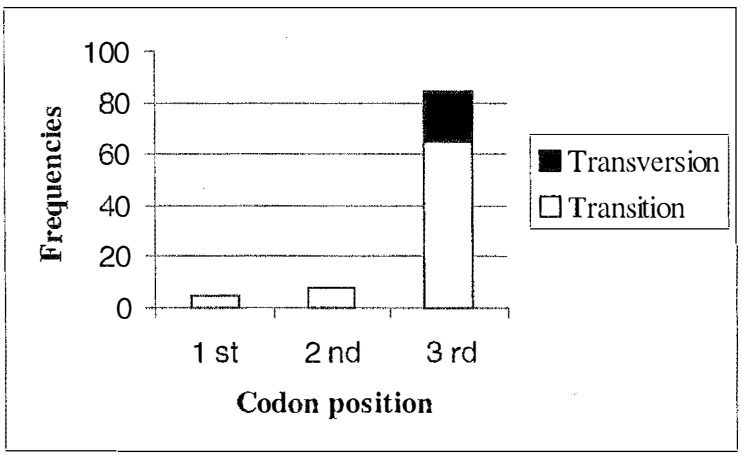


Figure 3. 12. Frequencies of transversion and transition and their codon positions whithin cytochrome *b* gene.

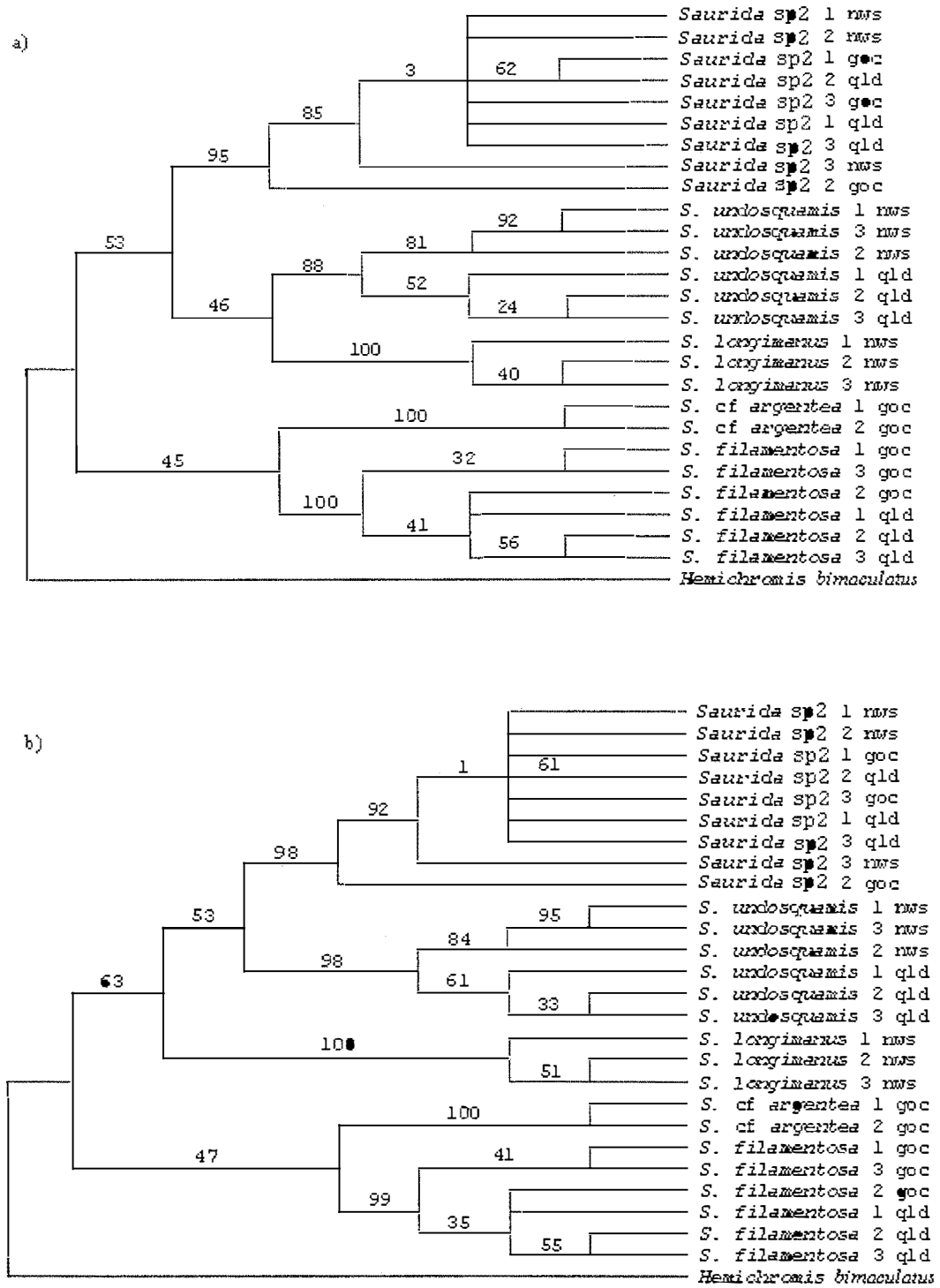


Figure 3. 13. Bootstrap 50% majority-rule consensus tree on cytochrome *b* gene; after species name are the location and sample's numbering; a) unweighted analysis, b) weighted to transversion=4 and transition=1, c) weighted to transversion alone.

Three weighting schemes were applied to the parsimony analysis namely unweighted, weighting of Tv:T1 at 3:1 and analysis of Tv only.

The unweighted maximum parsimony analysis of the cytochrome b gene (27 trees, 196 steps) is presented in Figure 3.13a. The topolgy of the unweighted maximum parsimony tree for cytochrome b differs from the result obtained with COI however, overall bootstrap support for this tree is lower than observed for COI. *Saurida undosquamis* and *S. longimanus* are grouped together as a clade with *Saurida* sp2, whilst *S.* cf *argentea and S. filamentosa* are again grouped as sister taxa in a separate assemblage. A 3:1 Tv:Ti weighted parsimony tree (24 trees, 290 steps) is presented in Figure 3.13b. *Saurida undosquamis* remains grouped with *Saurida* sp2 however, *S. longimanus* appears as a separate clade within the first assemblage, whilst *S.* cf *argentea and S. filamentosa* remain grouped as sister taxa.
Weighting transversions alone (11 trees, 46 steps, Figure 3.13c) saw high bootstrap support for the separation of the species and *S. longimanus* being placed basal to the other taxa. Otherwise the tree topology remained largely congruent except for the terminal branches where the loss of informative characters failed to resolve intraspecific geographical differences.

The DNADIST application of PHYLIP package was used to determine corrected distances based upon the Kimura 2-parameter model using a Ti:Tv ratio of 2:1. The result is a strong linear relationship between corrected distance and Ti and Tv frequency

3. 2c. Comparison of mitochondrial 12S RNA gene.

The same samples were used to determine the sequence identity of the 12sRNA gene with the addition of two further *S.* cf *argentea* individuals, two *S. undosquamis* and one each *of S. longimanus* and *Saurida* sp2.

Figure 3. 14. Proportion of corrected divergence (Kimura, 1980), frequencies of transversion and transition in cytochrome *b*.

The sequenced 12sRNA fragment was 389 bp in length (Figure 3.15). *Chalceus macrolepidotus* was chosen as an outgroup taxon for phylogenetic analysis following a BLAST search of the NCBI database.

Intraspecific variation was only recorded in *Saurida* sp2 and *S. undosquamis*. Interspecific distances ranged from 1.8% between *Saurida* sp2 and *S. undosquamis* to 5.5% between *S. filamentosa* and *S. longimanus*.

The Ti:Tv ratio determined from the 12sRNA gene was 2:1 which reflects the low level of sequence diversity observed in this gene fragment.

The result of an unweighted maximum parsimony analysis (4000 trees,97 steps) is presented in Figure 3.16a. The topology of this tree differed from the unweighted analyses of COI and cytochrome b in that *S. longimanus* formed an assemblage with *Saurida* sp2, albeit with low (44%) bootstrap suppoert. *Saurida filamentosa* and *S.* cf *argentea* were again grouped as sister taxa with strong bootstrap support however, the presence *of S. undosquamis* in the same assemblage was weakly supported (48%).

A 2:1 Tv:Ti weighted parsimony analysis (4 trees, 132 steps) is presented in Figure 3.16b.

Figure 3. 15. Partial sequence of mitochondrial 12S RNA; nws=North West Shelf, goc=Gulf of Carpentaria, QLD=Queensland; number after species name is the number of samples in each group; N=A, C, G, or T.

Saurida sp2 1 nws
Saurida sp2 2 nws
Saurida sp2 3 nws
Saurida sp2 4 nws
Saurida sp2 1 goc
Saurida sp2 2 goc
Saurida sp2 3 goc
Saurida sp2 1 qld
Saurida sp2 2 qld
Saurida sp2 3 qld
S. undosquamis 1 nws
S. undosquamis 2 nws
S. undosquamis 3 nws
S. undosquamis 4 nws
S. undosquamis 5 nws
S. undosquamis 1 qld
S. undosquamis 2 qld
S. undosquamis 3 qld
S. cf argegntea 1 goc
S. cf argegntea 2 goc
S. cf argegntea 3 goc
S. cf argegntea 4 goc
S. filamentosa 1 goc
S. filamentosa 2 goc
S. filamentosa 3 goc
S. filamentosa 1 qld
S. filamentosa 2 qld
S. filamentosa 3 qld
S. longimanus 1 nws
S. longimanus 2 nws
S. longimanus 3 nws
S. longimanus 4 nws
220
230
240
250
260
270
280
290
300
310
320
330
340
350
360
370
380
390
400

Saurida undosquamis is grouped with *Saurida* sp2 although bootstrap support is low (52%) and *S. longimanus* is placed basal to the other taxa. Only the *S. filamentosa* and *S.* cf *argentea* has strong bootstrap support. An analysis based upon Transversions only was deemed unnecessary due to the low level of sequence divergence and hence low number of informative characters in the 12sRNA gene.

Corrected distance values based upon the Kimura 2-parameter model determined by the DNADIST function of PHYLIP, were plotted against transition and transversion frequencies.

There is a clear linear relationship between Ti and Tv frequency and corrected distance (Figure 3.17).

3. 2d. Combined analysis of mitochondrial CO1, cytochrome b, and 12S RNA.

A subset of individuals that gave unequivocal sequences for all three genes were selected for a combined sequence analysis. The outgroup was a combined sequence derived from the three outgroup taxa used for the individual sequence analyses.

The result of the unweighted maximum parsimony analysis (3 trees, 485 steps) is given in Figure 3.18a. The species separation continues to have strong bootstrap support and two assemblages are apparent. The first assemblage is comprised of *Saurida* sp2 and *S. undosquamis* and has strong bootstrap support. The second assemblage includes *S. filamentosa* and *S.* cf *argentea* as sister taxa *with S. longimanus* basal to the second assemblage.

A weighted maximum parsimony analysis (3:1 Tv:Ti for COI and cytochrome b and 2:1 Tv:Ti for 12sRNA) resulted in three trees of 867 steps (Figure 3.18b). *Saurida* sp2 *and S. undosquamis* remain grouped as sister taxa as do *S. filamentosa* and *S.* cf *argentea* with both assemblages having high bootstrap support. *Saurida longimanus* is basal to both assemblages.

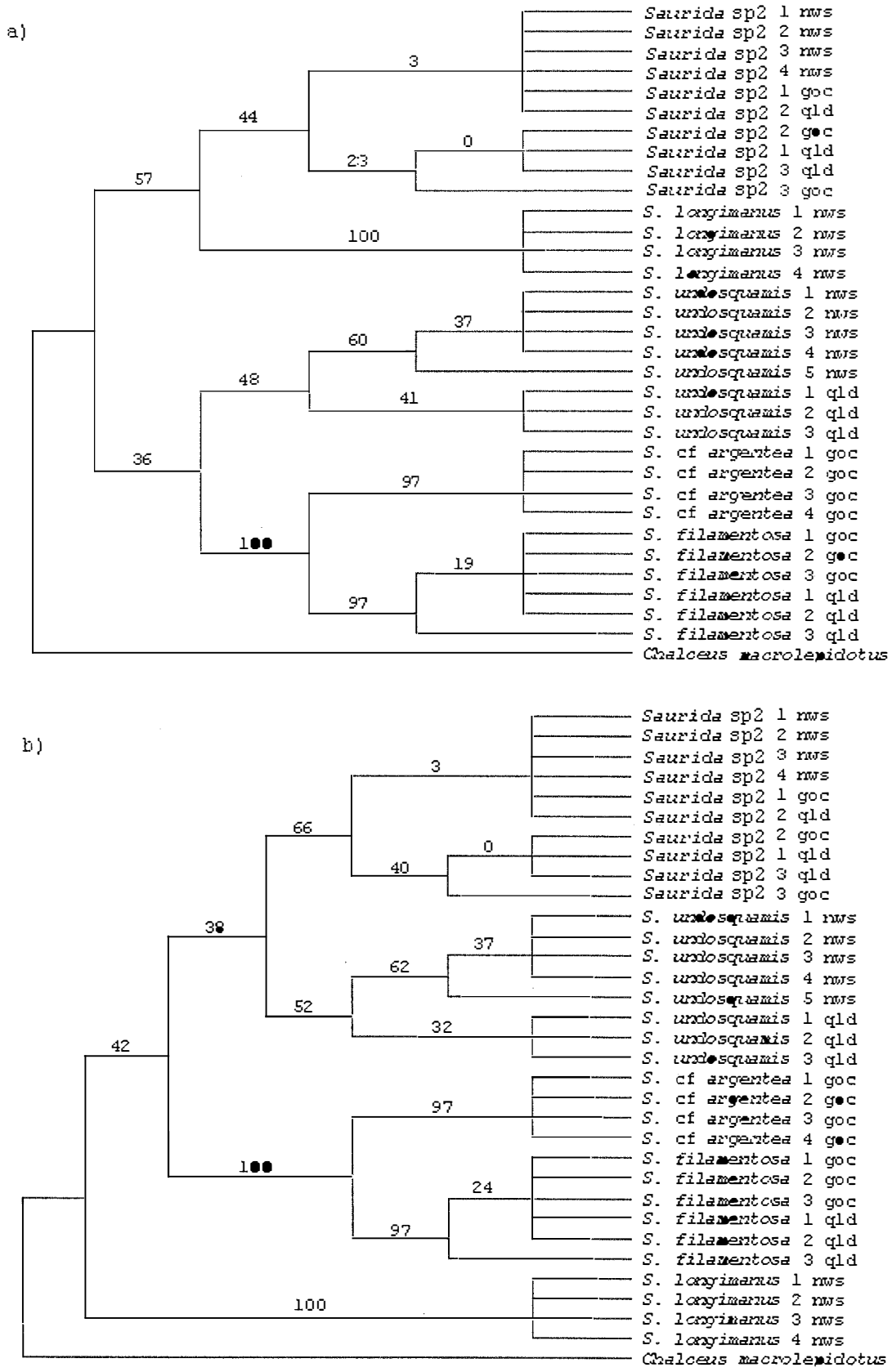


Figure 3. 16. Bootstrap 50% majority-rule consensus tree on 12S RNA; after species name are the location and sample's numbering; a) unweighted analysis, b) weighted to transversion=2, and transition=1.

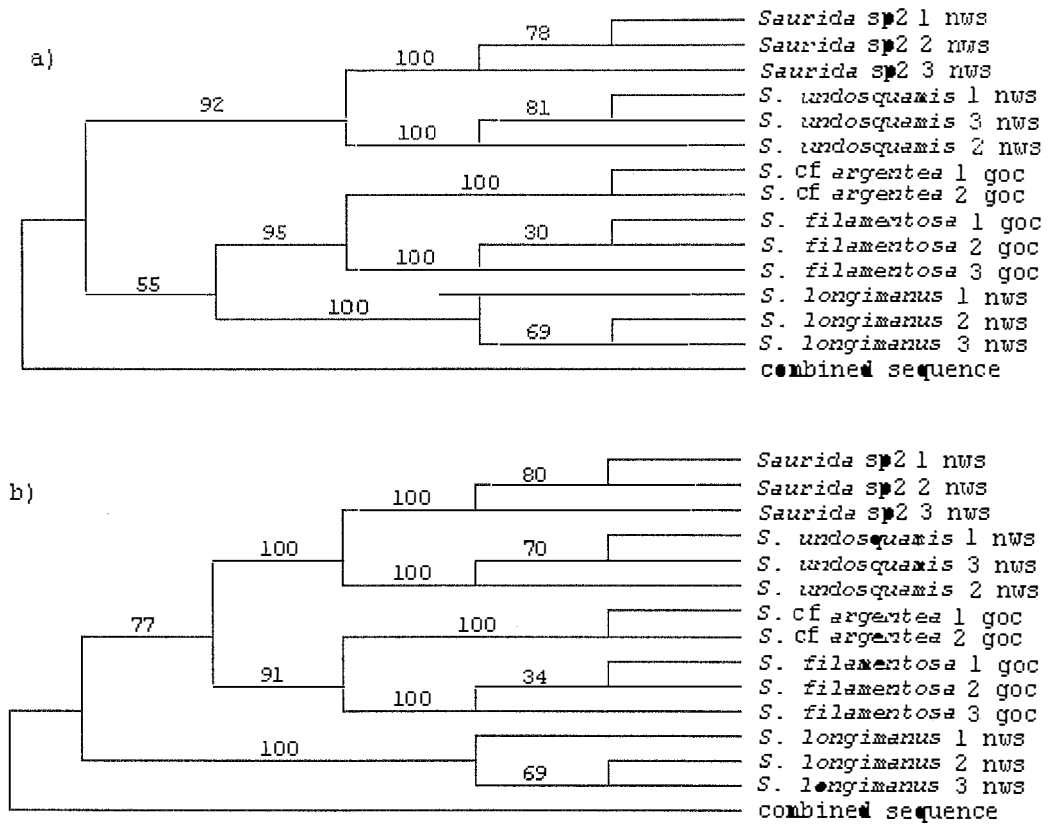


Figure 3. 18. Bootstrap 50% majority-rule consensus tree on the combined analysis under branch and bound search; after species name are the location and sample's numbering; a) unweighted analysis, b) unequally weighted to transversion (CO1, 3 to 1; cytochrome *b*, 4 to 1; 12S RNA, 2 to 1).

CHAPTER 4 GENERAL DISCUSSION

4. 1. Species integrity

The alpha taxonomy of lizardfish from the genus *Saurida* has been problematic due to their conservative morphology. An example of this is provided by the sympatric occurrence of *S. undosquamis* and *Saurida* sp2 in northern Australian waters. For a substantial period, these two taxa were considered a single species despite having different body sizes, patterns of sexual maturation and life histories (Thresher *et al.* 1986). *Saurida. undosquamis* reaches a large size (over 50 cm), matures sexually at a minimum size of 25-30 cm or at the age of 3-4 yr, and reaches a maximum age of just over 7 yr (Thresher *et al.* 1986). *Saurida* sp2 is a much smaller form with a maximum length of *c.* 25 cm. It matures sexually at a size of less than 8 cm or at the age 86 days (Thresher *et al.* 1986). It is effectively an annual species with only a very few individuals living longer than 12 months (Thresher *et al.* 1986).

In the study reported in this thesis, complete discrimination of all individuals from northern Australian waters was achieved by *LDH** locus, general protein patterns and one of the protein patterns, the *CK** locus, the *LDH**, general protein and *CK** electromorphs separated all samples into two, five and three groups respectively. Combining the three patterns above and morphotypes of known species can be used as a synoptic key for easy discrimination of saurids.

Of the five groups, there was a problem with the trivial identification of one form. This species was checked with the extensive fish collection held in the Munro Collection at the CSIRO Marine Laboratories, Hobart and was found to share more morphological characters with *S. argentea* than with other forms. However, it differed in the proportion of keel and body size. Since only relatively few fish were collected in this study and some were not in good morphological condition, it is difficult to confirm the specific status of the form. Currently thinking is that it is, in fact, *S. argentea* or is closely related to it; the working name *S.* cf *argentea* has been applied to this form (Last, pers. comm).

On the basis of the electromorph patterns, fish collected for use in this study have been allocated to one of five taxa: *S. undosquamis* (28.1% of fish sampled), *Saurida* sp2 (61.4%) (maybe synonymous with *S. grandisquamis,* research in progress, Last, pers. comm.), *S.* cf *argentea* (2.8%), *S. filamentosa* (2.7%), and *S. longimanus* (5%).

The slow pattern of *LDH** locus was typical of *S. undosquamis, S.* cf *argentea,* and *S. filamentosa* while the fast pattern of *LDH** locus was typical of *Saurida* sp2 and *S. longimanus.* The slow pattern of *CK** locus was typical of *S.* cf *argentea,* and *S. filamentosa,* the medium pattern of *CK** locus was typical of *S. undosquamis,* and *Saurida* sp2 and the fast pattern of *CK** locus was typical of *S. longimanus.* In addition to *LDH** and general protein patterns, other three fixed allelic differences were subsequently found between *Saurida* sp2. and *S. undosquamis*: *SOD**, *MDH**, and *AAT-2**.

The clear distinction of *Saurida* sp2. is contrary to the systematic study of saurids by Adjei (1984), especially in the specific status of *S. undosquamis* and *S. grandisquamis.* Adjei (1984) rejected *S. grandisquamis* and synonymized this species with *S. undosquamis* based on the examination of just one syntype.

Yamada and Ikemoto (1979), on the basis of morphology and ecology, recognized two distinct types of *S. undosquamis* in the East China Sea. Subsequent electrophoretic study by Yamaoka *et al.* (1989) showed that the two types have a genetic distance of 0.5582 that is within a range of differentiation at the species level. The two types did not share any alleles at the following loci: *AAT-2**, *ADH**, *FH-1**, *FH-2**, *GPI-2**, *SOD**, and *SP-2**. No specific status was given to the second species. Since these species were distinct morphologically and ecologically and share *LDH-3** (muscle tissues) and *MDH** loci but share no alleles at *GPI-2** locus, it is unlikely that the second species was *Saurida* sp2 (= *S. grandisquamis*?) in the present study. LDH is a tissue specific enzyme and it is coded by three gene loci: *LDH-1** (eye), *LDH-2** (heart) and *LDH-3** (muscles). In the present study, *LDH** locus was screened from muscle tissue, so it is likely to be the same as *LDH-3** in Yamaoka's study.

Saurida undosquamis can be separated from the other three species with the following characters. It is different from *S.* cf *argentea* by its lack of short pectoral fin, which does not reach beyond the base of the pelvic fin, 3 or 4 rows of teeth in outer palatine band, a centrally placed group of vomerine teeth. It can be separated from *S. filamentosa* by its lack of a filamentous second and sometimes third dorsal ray and from *S. longimanus* by its lack of a long pectoral fin that extends beyond origin of dorsal fin when laid back (Adjei, 1984).

The distribution of some species of saurids has been well studied in some areas. In Japanese waters, Yamaoka *et al.* (1989) recognized two different types of *S. undosquamis* that have different depth distribution. The southern type is found at depths to about 200 m at the edge of the continental shelf while the northern type always inhabits muddy bottoms less than 150 m deep. *Saurida argentea* occurs in estuarine or shallow water to a recorded depth of about 116 m. *Saurida filamentosa* occur in central Indo-Pacific at depth of about 200 m. *Saurida longimanus* occur at a depth of about 200 m (Adjei, 1984).

In the present study, the separation of the species in saurids samples based on allozyme data was strongly supported by mitochondrial DNA analysis. Relatively high bootstrap values were obtained from combined analysis of cytochrome oxidase *c* subunit 1 gene, cytochrome *b* gene and 12S RNA analysis. Individual analysis of the first two genes also provides strong bootstrap values. A decrease in bootstrap values was given in the analysis of 12S RNA alone although it still clustered the fish into five groups. This may result from the low proportion of divergence in 12S RNA as described in the pairwise comparison between taxa.

4. 2. Genetic variation in *Saurida* sp2. and *S. undosquamis*.

Both *Saurida* sp2. and *S. undosquamis* showed a relatively high genetic variation with the average heterozygosity of 8.7% for 12 loci and 7.2% for 11 loci, respectively. The average total heterozygosity for 195 species of marine and freshwater fish was 5.1% (Ward *et al.* 1994) and for 106 species of marine teleosts it was 5.5% (Smith and Fujio, 1982).

A low number of polymorphic loci were observed in the species examined here. This result maybe due to the low number of loci that were resolved in screening. Higher numbers of polymorphic loci were reported by Yamaoka *et al.* (1989) for a study of *S. undosquamis* in the East China Sea,. The southern and northern types of *S. undosquamis* had 7 and 13 polymorphic loci, respectively, under the 0.95 criterion.

In this study, evidence of genetic structuring of both *Saurida* sp2 and *S. undosquamis* across northern Australia was obtained. In the former species, a significant differentiation at the *MPI** locus was observed between the North West Shelf (NWS) and both Gulf of Carpentaria (GOC) and Queensland (QLD) samples. At the *MPI** locus, two common alleles (>0.05) were observed in all three samples and two and one rare alleles (<0.05) were observed in NWS and QLD, respectively. No rare allele was observed in GOC sample. In NWS sample, the two common alleles have a relatively similar frequency (0.475 and 0.497). Significant differentiation was also observed at the *ADA** locus between the NWS and QLD sample. In this locus, two common and four rare alleles were observed in NWS sample while a reverse result was obtained in QLD sample with four common and two rare alleles.

In *S. undosquamis,* evidence of temporal variation in NWS samples was observed at the *MDH** locus. Significant differentiation at the *MDH* locus* was also observed between NWS 1 and QLD and NWS 2 and QLD. At this locus, one, two and three common alleles were observed in NWS 1, NWS 2 and QLD samples, respectively. One rare allele was observed in NWS 1 and NWS 2 samples and no rare allele was observed in QLD sample. Other locus that showed significant differentiation were *GPI-2** and *MPI** between NWS 1 and QLD and NWS 2 and QLD. At the *MPI** locus, two common alleles were observed in NWS 1 and QLD samples and one common allele was observed in NWS 2 sample. One rare allele was observed in NWS 1 sample, three rare alleles were observed in NWS 2 sample and no rare allele was observed in QLD sample. At the *GPI-2** locus, two common alleles were observed in NWS 1 and NWS 2 samples. One rare allele was observed in NWS 1 sample and no rare allele was

observed in NWS 2 sample. In QLD sample, the *GPI-2** locus was fixed to the common allele.

The mean *G*ST values (uncorrected for sampling error) were 0.063 (for 8 variable loci) and 0.098 (for 11 loci) for *Saurida* sp2. and *S. undosquamis* respectively. The average (similarly uncorrected) value reported for 57 species of marine teleosts was 0.062 (Ward *et al.* 1994). Genetic diversity in *Saurida* sp2. from the three samples is relatively similar to most other marine fishes thus far studied while in *S. undosquamis,* it is towards the higher end of values reported for other taxa.

The genetic differentiation in *Saurida* sp2. across northern Australian waters based on allozyme data was not, however, supported by mitochondrial DNA sequence data. Only small samples (3-4 individuals per sample) were used for checking the geographical variation among samples. In *S. undosquamis*, the evidence of population structuring across northern Australian waters from allozyme data was strongly supported by mitochondrial DNA sequence data. NWS population differed from QLD population.

As a consequence of a reduction in population size of many species during the last glaciation, most species show less genetic variation (Nei and Graur, 1984). This result from the bottleneck effect in population size that may endure for hundreds of years after the recovery of population size (Nei *et al.* 1975). To understand the population structure of both *S. undosquamis* and *Saurida* sp2 across northern Australia, it is important to understand the geological and sea-level changes of the habitat of the fish.

Deep water in North West Shelf and Queensland waters were separated by a shallower water in Gulf of Carpentaria. While the first two areas has a depth of around 1000 m, the later is an epicontinental sea that lies between Australia and Papua New Guinea with a maximum depth of ~69 m and is connected to the Arafura Sea across a ~53-m sill. In the east, it is connected to the Coral Sea through Torres Straits with a depth of ~12 m (Torgersen *et al.* 1985).

Historically, Australia and New Guinea were connected by a dry land which was formed 120 000 to 115 000 years BP. During the low sea level of the last glacial maximum and a hydrological balance, Lake Carpentaria existed. This lake basin was isolated from the marine environment by a sill that last above sea level from 75 000 to 10 000 years BP. The Carpentaria basin was a marine to brackish water body >36 kyr BP, fresh to brackish water 26-36 kyr, before returning to brackish to saline water 10-26 kyr. The marine conditions were gradually restored with the rise of sea level in Arafura Sea and then the Carpentaria basin ~10kyr BP (Torgersen *et al.* 1985).

The shallowest connection between Australia and New Guinea is in the Torres Strait. This area prevented sea water exchange between Coral Sea in the east and Arafura Sea in the west remained closed for about 100 000 years and reopened about 8000 to 6500 years BP (Galloway and Kemp 1981; Torgersen *et al.* 1985).

This variation in sea levels effectively isolated marine species in the area across northern Australia into western and eastern allopatric populations.

These separation events should be adequate to explain the population structuring reports here for the saurid species. Even today in the Torres Strait, strong tidal current that driven by large sea-level differences (up to 6 m) predominate. This results from the incoherent tides on either side of the strait (Wolanski *et al.* 1988). These may serve to provide a partial on-going barrier between western and eastern areas.

Subdivisions of marine populations by land bridges or by hydrological factors resulting in genetic subdivision have been widely reported elsewhere. Avise (1992) illustrates examples from a range of marine tax where distinct genetic subdivision occurs west and east of the Florida Peninsula.

Evidence of population structure in other species was also noticed across northern Australia. Benzie *et al.* (1992) reported that populations of *Penaeus monodon* demonstrated highly significant differences in gene frequencies between the west-coast population and those on the northern and eastern coastlines. Genetic diversity reduced

from east to west indicating that population from the east colonized the northern and western coasts after the last opening of the Torres Strait.

In a study of coastal Australian barramundi, Keenan (1994) demonstrated that populations from the western and southern Gulf of Carpentaria differed genetically from populations of the north-eastern Gulf and east coast of Queensland. The migration of genes happened from east-to-west through the Torres Strait. The genetic diversity reduced as the population spread into new habitat gave a 'one dimensional stepping stone' migration model. Elliott (1996) demonstrated that there was little or no gene flow, in *Lutjanus malabaricus,* through Torres Strait since its opening about 8000 years ago. The mitochondrial DNA analysis suggested that there were multiple stocks of the fish (NWS, GOC, QLD).

The number of migrants per generation in both *Saurida* sp2.(<4) and *S. undosquamis* (<1) in the present study were relatively low. Wright (1931) demonstrated that populations will be likely to diverge from the source if the number of migrants per generation is less than 1. One or more individual exchange per generation will be sufficient to prevent genetic differentiation through gene flow and genetic drift. This calculation provides only a rough estimate since it ignores any effects of natural selection.

In samples collected for this study, *Saurida* sp2 was more abundant than *S.* undosquamis. This accords with the results of Thresher *et al.* (1986) who studied the population structure of lizardfish from North West Shelf waters. They observed that during a 5 year period (1978-1983), the population size of *Saurida* sp2 increased rapidly, nearly doubling every year; this was far greater than the rate of increase in numbers of *S. undosquamis.* The result suggested that reproductive success has been high every year.

Smith and Fujio (1982) suggest that species with large population size are likely to occupy a wider range while species with less population size are likely to occupy narrow range. Such a notion conforms to the greater abundance of *Saurida* sp2. and the

lower population size of *S. undosquamis*. While the former occurred in three samples (NWS, GOC, QLD), the later occurred only in NWS and QLD samples. No sample of *S. undosquamis* was found from the Gulf of Carpentaria fish. The lack of this fish in this area was also observed by Blaber *et al.* (1994) in a study of distribution, biomass and community structure of demersal fishes in this area. Unlike *S. undosquamis*, they found that *Saurida* sp2 were widely distributed throughout the Gulf. Although population size may correlate with the range of the species, the effective population sizes in marine species are unknown and it seems to have little influence on marine teleost heterozygosities (Smith and Fujio, 1982).

The evidence of population structuring in northern Australian waters has an important implication for the management of fisheries in this area. However, confirmation of the proposed genetic differentiation need more detailed work that include an extension of the sampling range to Indonesian waters that close to Australian waters and have a lack of barriers to gene flow. Also, sample from Japanese waters need to be examined. In addition, more mitochondrial DNA works, such as restriction analysis is required with sufficient sample sizes for population studies.

4. 3. Phylogenetic relationships

There are some difficulties in the systematic studies of *Saurida* due to the morphological similarities that are compounded by their widespread distribution. Many meristic characters, such as lateral-line scales and fin rays have a number that are overlapped between many of the species (Adjei, 1984).

In this study, direct sequencing of mitochondrial DNA has been used to clarify the phylogenetic relationship in some species of saurids. The number of taxa used for analysis was low with only 5 species being available. PAUP analysis showed that *S. undosquamis* is the sister taxon of *Saurida* sp2. This results accorded with the morphological similarities in most characters between the two species. Similarly, *S.* cf *argentea* is the sister taxon of *S. filamentosa.*

One inconsistency was found in the relationship of *S. longimanus* with the other four taxa. In the systematic studies of saurids from Australian waters, Adjei (1984) grouped *S. longimanus* together with *S. undosquamis*. This is not fully supported here.

The results of the present phylogenetic analysis suggest that the direct sequencing of mitochondrial DNA provide a powerful tool to study genetic relationships in saurids. However, a far more detailed study would be required with far more species of saurids being included. Also, the use of outgroup that is closely related to *Saurida* species is required. Since there is a lack of appropriate outgroup in this study, the outgroup was chosen from the reference taxa from a BLAST search. In the combined analysis (cytochrome oxidase *c* subunit 1 gene, cytochrome *b* and 12S RNA), the outgroup was a combined sequence from the reference taxa.

In the present study, the genus *Saurida* was placed under the Family Synodontidae. However, in the near future, there will be a tendency to place *Saurida* species under the Family Harpadontidae following Johnson *et al.* (1996) especially in the study of saurids in Australian waters (Last, pers. comm.).

REFERENCES

Adjei, E. L. (1984). *The Systematics of Indo-Pacific* Saurida *and* Trachinochepalus *(Pisces: Synodontidae) and the biology of four local species.* PhD thesis, University of Queensland, St Lucia.

Agnese, J. F., Adepogourene, B., Abban, E. K., and Fermon, Y. (1997). Genetic differentiation among natural populations of the Nile tilapia *Oreochromis niloticus* (Teleostei, Cichlidae). *J. Hered.,* **79**: 88-96.

Altukhov, Y. P. (1981). The stock concept from the viewpoint of population genetics. *Can. J. Fish. Aquat. Sci.,* **38**: 1523-1538.

Anderson, W.D., and Heemstra, P. C. (1980). Two new species of western Atlantic *Anthias* (Pisces: Serranidae), redescription of *A. asperilinguis* and review of *Holanthias martinicensis. Copeia,* **1980**: 72-87.

Andersson, B., and Gibbs, R. A. (1994). PCR and DNA sequencing. **In** *The Polymerase Chain Reaction,* Ed. K. B. Mullis, F. Ferré, R. A. Gibbs, pp 202-213, Birkhauser, Boston.

Arculeo, M., Mauro, A., Scelsa, G., Lobrutto, S., Cammarata, M., and Parrinello, N. (1996). Protein differences among the Mediterranean species of the genus *Spicara. J. Fish Biol.,* **49**: 1317-1322

Asahida, T., Yamashita, Y., and Kobayashi, T. (1997). Identification of consumed stone flounder, *Kareius bicoloratus* (Basilewsky), from the stomach contents of sand shrimp, *Crangon affinis* (De-Haan) using mitochondrial DNA analysis. *J. Exp. Mar. Biol. Ecol.,* **217**: 153-163.

Avise, J. C. (1974). Systematic value of electrophoretic data. *Syst. Zool.,* **23**: 4-81.

Avise, J.C. (1992). Molecular population structure and the biogeographic history of a regional fauna : history with lessons for conservation biology. *Oikos* **63**: 62-79.

Avise, J. C. (1994). *Molecular Markers, Natural History and Evolution.* Chapman & Hall, New York.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A., and Saunders, N. C. (1987). Instranspecific phylogeography: the mithocondrial DNA bridge between population genetics and systematics. *Ann. Rev. Ecol. Syst.,* **18**:

489-522.

Avsar, D., Bingel, F., and Ünsal, M. (1987). Application of Mahalanobis distance function for the morphometric separation of lizardfish (*Saurida undosquamis* Richardson) stocks in the Gulf of Mersin. *Metu J. Pure App. Sci.,* **20**: 311-320.

Baldwin, C. C., Johnson, G. D., Paxton, J. R. (1997). *Protoblepharon rosenblatti*, a new genus and species of flashlight fish (Beryciformes, Anomalopidae) from the tropical South Pacific, with comments on anomalopid phylogeny. *Proc. Biol. Soc. Wash.,* **110**: 373-383.

Bartlett, S. E., and Davidson, W. S. (1991). Identification of *Thunnus* tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome *b* genes. *Can. J. Fish. Aquat. Sci.,* **48**: 309-317.

Barton, N., and Jones, J. S. (1983). Mitochondrial DNA: new clues about evolution. *Nature,* **306**: 317-18.

Benzie, J. A. H., Frusher, S., and Ballment, E. (1992). Geographical variation in allozyme frequencies of populations of *Penaeus monodon* (Crustacea: Decapoda) in Australia. *Aust. J. Mar. Freshwat. Res.,* **43**: 715-25.

Birky, C. W. (1983). Relaxed cellular controls and organelle heredity. *Nature,* **222**: 468-475.

Blaber, S. J. M., Brewer, D. T., and Harris, A. N. (1994). Distribution, biomass and community structure of demersal fishes of the Gulf of Carpentaria, Australia. *Aust. J. Mar. Freshwat. Res.,* **45**: 375-96.

Bremer, K., and Wanntorp, P. H. E. (1979). Geographic populations or biological species in phylogeny reconstruction. *Syst. Zool.,* **28**: 220-224.

Brewer, G. J., and Sing, C. F. (1970). *An Introduction to Isozyme techniques.* Academic Press, New York.

Brown, W. M., George, M., and Wilson, A. C. (1979). Rapid evolution of animal mitochondrial DNA. *Proc. Nat. Acad. Sci. U. S. A.,* **76**: 1967-1971.

Bruno, T. J. (1991). *Chromatographic and Electrophoresis Methods.* Prentice-Hall, Inc., New Jersey.

Bryden, L. J., Denovanwright, E. M., and Wright, J. M. (1998). RON-1 SINES - a tRNA-derived, short interspersed repetitive DNA element from *Oreochromis*

niloticus and its species-specific distribution in old world cichlid fishes. *Mol. Mar. Biol. Biotech.*, **7**: 48-54.

Buth, D. G. (1984). The application of electrophoretic data in systematic studies. *Ann. Rev. Ecol. Syst.*, **15**: 501-522.

Buth, D. G. (1990). Genetic principles and the interpretation of electrophoretic data **In** *Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management*. Ed. D. H. Whitmore, pp. 1-21, CRC Press, Boca Raton.

Carlson, S. S., Wilson, A. C., and Maxon, R. D. (1978). Do albumin clocks run on time ? A reply. *Science*, **200**: 1183-1185.

Carr, S. M., and Marshall, D. (1991). Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome *b* gene of Atlantic cod (*Gadus morhua*) detected by the polymerase chain reaction. *Can. J. Fish. Aquat. Sci.*, **48**: 48-52.

Carvalho, G. R., and Hauser, L. (1994). Molecular genetics and the stock concept in fisheries. *Rev. Fish Biol. Fish.*, **4**: 326-350.

Chow, S. (1993). PCR-RFLP analysis on thirteen western Atlantic snappers (Subfamily Lutjaninae): a simple method for species and stock identification. *Fish. Bull.*, **91**: 619-627.

Compagno, L. J. V. (1977). Phyletic relationships of living sharks and rays. *Am. Zool.*, **17**: 303-322.

Creech, S. (1991). An electrophoretic investigation of populations of *Atherina boyeri* Risso, 1810 and *A. presbyter* Cuvier, 1829 (Teleostei: Atherinidae): genetic evidence in support of the two species. *J. Fish Biol.*, **39**: 807-816.

Creech, S. (1992). A multivariate morphometric investigation of *Atherina boyeri* Risso, 1810 and *Atherina presbyter* Cuvier, 1829 (Teleostei: Atherinidae): morphometric evidence in support of the two species. *J. Fish Biol.*, **41**: 341-353.

Didier, D. A., Leclair, E. E., and Vanbuskirk, D. R. (1998). Embryonic staging and external features of development of the chimaeroid fish, *Callorhinchus milli* (Holocephali, Callorhinchidae). *J. Morph.*, **236**: 25-47.

Eastman, J. T. (1997). Phyletic divergence and specialization for pelagic life in the Antarctic nototheniid fish *Pleuragramma antarcticum*. *Comp. Biochem. Phys.*, **118**: 1095-1101.

Elliott, N. G., and Ward, R. D. (1992). Enzyme variation in orange roughy, *Hoplostethus atlanticus* (Teleostei: Trachichthydae), from southern Australian and New Zealand waters. *Aust. J. Mar. Freshwat. Res.,* **43**: 1561-1571.

Elliott, N. G . (1996). Allozyme and mitochondrial DNA analysis of the tropical saddle-tail sea perch, *Lutjanus malabaricus* (Schneider), from Australian waters. *Mar. Freshwat. Res.,* **47**: 869-876

Elliott, N. G., Haskard, K., and Koslow, J.A . (1995). Morphometric analysis of orange roughy (*Hoplostethus atlanticus*) off the continental slope of southern Australia. *J. Fish Biol.,* **46**: 202-220.

Elliott, N. G., and Ward, R. D. (1992). Enzyme variation in orange roughy, *Hoplostethus atlanticus* (Teleostei : Trachichthydae), from southern Australian and New Zealand waters. *Aust. J. Mar. Freshwat. Res.,* **43**: 1561-1571.

Elliott, N. G., and Ward, R. D. (1994). Enzyme variation in jackass morwong, *Nemadactylus macropterus* (Schneider, 1801) (Teleostei: Cheilodactylidae), from Australian and New Zealand waters. *Aust. J. Mar. Freshwat. Res.,* **45**: 51-67.

Elliott, N. G., and Ward, R. D. (1995). Genetic relationships of eight species of Pacific tunas (Teleostei, Scombridae) inferred from allozyme analysis. *Mar. Freshwat. Res.,* **46**: 1021-1032.

Elo, K., Ivanoff, S., Vuorinen, J. A., and Piironen, J. (1997). Inheritance of RAPD markers and detection of interspecific hybridization with brown trout and Atlantic salmon. *Aquaculture,* **152**: 55-65.

Erlich, H. A., Gelfand, D.H., and Saiki, R. K. (1988). Specific DNA amplification. *Nature,* **331**: 461-462.

Fairbairn, D. J. (1981a). Biochemical genetic analysis of population differentiation in Greenland halibut (*Reinhardtius hippoglossoides*) from the Northwest Atlantic, Gulf of St. Lawrence, and Bering Sea. *Can. J. Fish. Aquat. Sci.,* **38**: 669-677.

Fairbairn, D. J. (1981b). Which witch is which? A study of the stock structure of witch flounder (*Glyptocephalus cynoglossus*) in the Newfoundland region. *Can. J. Fish. Aquat. Sci.,* **38**: 782-794.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution,* **39**: 783-791.

Ferguson, A. (1980). *Biochemical Systematics and Evolution.* John Wiley and Son, New York.

Fevolden, S. E., Pogson, G. H. (1997). Genetic divergence at the synaptophysin (syp i) locus among Norwegian coastal and north-east Arctic populations of Atlantic cod. *J. Fish Biol.,* **51**: 895-90.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotech.,* **3**: 294-299.

Galloway, R. W., and Kemp, E. M. (1981). Late Cainozoic environments in Australia. In '*Ecological Biogeography of Australia*'. (Ed. A. Keast.) pp. 51-80. Junk: The Haque.

Gelfand, D. H. (1989). Taq DNA Polymerase. **In** *PCR Technology: Principles and Applications for DNA Amplification*, Ed. Erlich, H. A., pp 17-22. Stockton Press, New York.

Golani, D. (1993). The biology of the Red Sea migrant, *S. undosquamis* in the Mediterranean and comparison with the indigenous confamilial *Synodus saurus* (Teleostei: Synodontidae). *Hydrobiologia,* **271**: 109-117.

Grant, W. S. (1984). Biochemical population genetics of Atlantic herring, *Clupea harengus. Copeia* **1984**: 357-364.

Grant, W. S. (1985). Biochemical genetic and stock structure of the south African anchovy, *Engraulis capensis* Gilchrist. *J. Fish Biol.,* **27**: 23-29.

Grant, W. S., and Stahl, G. (1988). Evolution of Atlantic and Pacific cod: loss of genetic variation and gene expression in Pacific cod. *Evolution,* **42**: 138-146.

Grant, W. S., Teel, D. J., Kobayashi, T., and Schmitt, C. (1984). Biochemical population genetics of Pacific halibut (*Hippoglossus stenolepis*) and comparison with Atlantic halibut (*H. hippoglossus*). *Can. J. Fish. Aquat. Sci.,* **41**: 1083-1088.

Grant, W. S., and Utter, F. M. (1980). Biochemical genetic variation in walleye pollock, *Theragra chalcogramma*: population sturcture in the outheastern Bering Sea and the Gulf of Alaska. *Can. J. Fish. Aquat. Sci.,* **37**: 1093-1100.

Grant, W. S., and Utter, F. M. (1984). Biochemical populations genetics of Pacific herring (*Clupea pallasi*). *Can. J. Fish. Aquat. Sci.,* **41**: 856-864.

Grant, W. S., Zhang, C. I., Kobayashi, T., and Stahl, G. (1987). Lack of genetic stock discretion in Pacific cod (*Gadus macrocephalus*). *Can. J. Fish. Aquat. Sci.*, **44**: 490-498.

Grewe, P. M., Krueger, C. C., Aquadro, C. F., Bermingham, E., Kincaid, H. L., and May, B. (1993). Mitochondrial DNA variation among lake trout *(Salvelinus namaycush)* strains stocked into Lake Ontario. *Can. J. Fish. Aquat. Sci.*, **50**: 2397-2403.

Grewe, P. M., Smolenski, A. J., and Ward, R. D. (1994). Mitochondrial DNA diversity in jackass morwong (*Nemadactylus macropterus*, Teleostei) from Australian and New Zealand waters. *Can. J. Fish. Aquat. Sci.*, **51**: 1101-1109.

Haddon, M., and Willis, T. J. (1995). Morphometric and meristic comparison of orange roughy (*Hoplostethus atlanticus*: Trachichthyidae) from the Puysegur Bank and Lord Howe Rise, New Zealand, and its populations from stock structure. *Mar. Biol.*, **123**: 19-27.

Hames, B. D. (1990). One-dimensional polyacrylamide gel electrophoresis **In** *Gel Electrophoresis of Proteins, a Practical Approach.* Ed. B. D. Hames and D. Rickwood, pp. 1-147, Oxford University Press, New York.

Hartl, D. L. (1991). *Basic Genetics.* 2nd Ed. Jones and Bartlett Publishers, Boston.

Hebert, P. D. N., and Beaton, M. J. (1989). 'Methodologies for allozyme analysis using cellulose acetate Electrophoresis.' (Helena Laboratories: Beaumont, TX.)

Heller, M. J. (1994). Fluorescent detection methods for PCR analysis. **In** *The Polymerase Chain Reaction*, Ed. K. B. Mullis, F. Ferré, R. A. Gibbs, p134-141, Birkhauser, Boston.

Henault, M., and Fortin, R. (1989). Comparison of meristic and morphometric characters among spring- and fall-spawning ecotypes of cisco (*Coregonus artedii*) in southern Quebec, Canada. *Can. J. Fish. Aquat. Sci.*, **46**: 166-173.

Hillis, D. M. (1987). Molecular versus morphological approaches to systematics. *Ann. Rev. Ecol. Syst.*, **18**: 23-42.

Hochberg, Y. (1988). A sharper Bonferroni procedure for multiple tests of significance. *Biometrika*, **75**: 800-802.

Hunter. R. L., and C. L. Market. (1957). Histochemical demonstration of enzymes

separated by zone electrophoresis in starch gels. *Science,* **125**: 1294-1295.

Iles, T. D., and Sinclair, M. (1982). Atlantic herring: stock discreteness and abundance. *Science,* **215** : 627-633.

Jeffreys, A.J., Royle, N.J., Wilson, V. and Wong, Z. (1988). Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. *Nature, Lond.,* **332**, 278-81.

Jeffreys, A.J., Wilson, V. and Thein. S.L. (1985). Hypervariable "minisatellite" regions in human DNA. *Nature, Lond.,* **314**, 67-73.

Jerry DR. (1997). Population genetic structure of the catadromous Australian bass from throughout its range. *J. Fish Biol.,* **51**: 909-920.

Johnson, G. D., Baldwin, C. C., Okiyama, M., and Tominaga, Y. (1996). Osteology and relationships of *Pseudotrichonotus altivelis* (Teleostei, Aulopiformes, Pseudotrichonotidae). *Ichhyol. Res.,* **43**: 17-45.

Johnson, M. S., Hebbert, D. R., and Moran, M. J. (1993). Genetic analysis of populations of north-westerrn Australian fish species. *Aust. J. Mar. Freshwat. Res.,* **44**: 673-8

Karotam J., Boyce T. M., Oakeshott J. G. (1995). Nucleotide variation at the hypervariable esterase 6 isozyme locus of *Drosophila simulans*. Mol. Biol. Evol. 12 : 113-122.

Keenan, C. P. (1991). Phylogeny of Australian species of flatheads (Teleostei, Platycephalidae) as determined by allozyme electrophoresis. *J. Fish Biol.,* **39**(suppl. A): 237-249.

Keenan, C. P. (1994). Recent evolution of population structure in Australian barramundi, *Lates calcarifer* (Bloch): an example of isolation by distance in one dimension. *Aust. J. Mar. Freshwat. Res.,* **45**: 1123-1148.

Kelsch, S. W., and Hendricks, F. S. (1986). An electrophoretic and multivariate morphometric comparison of the American catfishes *Ictalurus lupus* and *I. punctatus. Copeia,* **1986**: 646-652.

Kinsey, S. T., Orsoy, T., Bert, T. M., and Mahmoudi, B. (1994). Population structure of the Spanish sardine, *Sardinella aurita*: natural morphological variation in a genetically homogeneous population. *Mar. Biol.,* **118**: 309-318.

Lacson, J. M., and Bassler, C. P. (1992). Biochemical systematics of fishes of the genus *Stegastes* (Pomacentridae) from the Southern Marianas. *Mar. Biol.*, **113**: 457-462.

Landry, P. A., and Lapointe, F. J. (1996). RAPD problems in phylogenetics. *Zool. Scripa.*, **25**: 283-290.

Lee, S. C., Tsoi, S. C. M., Cheng, H. L., and Chang, J. T. (1997). Identification of *Anguilla japonica* and *A. marmorata* elvers by allozyme electrophoresis. *J. Fish Biol.*, **51**: 208-210.

Leis, J. M., and Renis, D. S. (1983). *The Larvae of Indo-Pacific Coral Reef Fishes.* University of Hawaii, Press. Honolulu.

Lessios, H. A. (1992). Testing electrophoretic data for agreement with Hardy-Weinberg expectations. *Mar. Biol.* **112**: 517-23.

Lewis, P. O., and Snow, A. A. (1992). Deterministic paternity exclusion using RAPD markers. *Mol. Ecol.*, **1**: 155-160.

Lewontin R. C. (1991). 25 years ago in genetics - electrophoresis in the development of evolutionary genetics - milestone or millstone.
Genetics 128: 657-662

Lockwood, S. F., Dillinger Jr., R. E., Birt, T. P. Green, J. M., and Snyder, T. P. (1993). Phylogenetic relationships among members of the Coregoninae inferred from direct sequencing of PCR- amplified mitochondrial DNA. *Can. J. Fish. Aquat. Sci.*, **50**: 2112-2118.

Lowry, P. S., Elliott, N. G., Yearsley, G. K., and Ward, R. D. (1996). Genetic variation and phylogenetic relationships of seven oreo species (Teleostei, Oreosomatidae) inferred from allozyme analysis. *Fish. Bull.*, **94**: 692-706.

Matsubara, K., and Iwai, T. (1951). Comparative study of the lizard-fishes referred to the genus *Saurida* found in the waters of Japan and China. *Mem. Agric. Kyoto Univ.*, **59**: 19-30.

Maxam, A. M.,and Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Nat. Acad. Sci., USA* **74**: 560-564.

McKay SJ. Smith MJ. Devlin RH. (1997). Polymerase chain reaction-based species identification of salmon and coastal trout in British Columbia. *Mol. Mar. Biol. Biotech.*, **6**: 131-140.

Meneezes, M. R. (1994). Little genetic variation in the oil sardine, *Sardinella langiceps* Val., from the western coast of India. *Aust. J. Mar. Freshwat. Res.*, **45**: 257-64.

Miller, P. J., Serventi, M., Soregaroli, D., Torricelli, P., and Gandolfi, G. (1994). Isozyme genetics and the phylogeny of Italian freshwater gobies (Teleostei, Gobioidei). *J. Fish Biol.*, **44**: 439-451.

Milton, D. A., and Shaklee, J. B. (1987). Biochemical genetics and population structure of blue grenadier, *Macruronus novaezelandiae* (Hector) (Pisces: Merluccidae), from Australian waters. *Aust. J. Mar. Freshwat. Res.*, **38**: 727-742.

Miracle, A. L., and D. E. Campton. (1995). Tandem repeat sequence variation and Length heteroplasmy in the mitochondrial DNA D-loop of the threatened Gulf of Mexico sturgeon, *Acipencer oxyrhynchus desotoi. J. Hered.*, **86**: 22-27.

Miya, M., and Nishida, M. (1996). Molecular phylogenetic perspective on the evolution of the deep-sea fish Genus *Cyclothone* (Stomiiformes, Gonostomatidae). *Icht. Res.*, **43**: 375-398.

Moran, P., and Dightman, D. A., Waples, R. S., and Park, L. K. (1997). PCR-RFLP analysis reveals substantial population-level variation in the introns of Pacific salmon (*Oncorhynchus* spp.). *Mol. Mar. Biol. Biotech.*, **6**: 315-327.

Moritz, C., Dowling, T. E., and Brown, W. M. (1987). Evolution and animal mitochondrial DNA: relevance for population biology and systematics. *Ann. Rev. Ecol. Syst.*, **18**: 269-292.

Morizot, D. C., and Schmidt, M. E. (1990). Starch gel electrophoresis and histochemical visualization of proteins **In** *Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management,* Ed. D. H. Whitmore, pp. 23-80, CRC Press Inc., Boca Raton.

Morizot, D. C., and Siciliano, M. E (1984). Gene mapping in fishes and other verterbrates. In *Evolutionary Genetics of Fishes.* Ed. By B. J. Turner, pp 173-234, Plenum, New York.

Mork, J., and Haug, T. (1983). Genetic variation in in halibut, *Hippoglossus hippoglossus* (L.), from Norwegian waters. *Hereditas* **98**: 167-174.

Mork, J., Ryman, R., Stahl, G., Utter, F., and Sundnes, G. (1985). Genetic variation in Atlantic cod (*Gadus morhua*) throughout its range. *Can. J. Fish. Aquat. Sci.*, **42**: 1580-1587.

Mulley, J. C., and Latter, B. D. H. (1981). Geographic differentiation of tropical Australian prawn populations. *Aust. J. Mar. Freshwat. Res.*, **32**: 897-906.

Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Sci. Am.*, **262**: 36-43.

Murata, S., Takasaki, N., Saitoh, M., and Okada, N. (1993). Determination of the phylogenetic relationships among Pacific salmonids by using short interspersed elements (SINEs) as temporal landmarks of evolution. *Proc. Nat. Acad. Sci. USA*, **90**, 6995-6999.

Murphy, W. J., and Collier, G.E. (1997). A molecular phylogeny for aplocheiloid fishes (Atherinomorpha, Cyprinodontiformes) - the role of vicariance and the origins of annualism. *Mol. Biol. Evol.*, **14**(8): 790-799.

Nei, M. (1973). Analysis of gene deversity in subdivided populations. *Proc. Natl. Acad. Sci. U. S. A.*, **70**: 3321-3323.

Nei, M. (1971). Interspecific gene differences and evolutionary time estimated from electrophoretic data on protein identity. *Am. Nat.*, **105**: 385-398.

Nelson, G. (1978). Ontogeny, phylogeny, paleontology, and the biogenetic law. *Syst. Zool.*, **27**: 324-45.

Norman, J. R. (1935). A revision of the lizard-fishes of the genera *Synodus*, *Trachinochepalus*, and *Saurida. Proc. Zool. Soc. Lond.*, **1**: 99-135.

Olivo, P. D., Van De Walle, M. J., Laipis, P. J., and Hauswirth, W. W. (1983). Nucleotide sequence evidence for rapid genotypic shifts in the bovine mitochondrial DNA D-loop. *Nature,* **306**: 400-402.

Orti, G., Bell, M. A., Reimchen, T. E., and Meyer, A. (1994). Global survey of mitochondrial DNA eequences in the threespine stickleback: evidence for recent migrations. *Evolution,* **48**: 608-622.

Oste, C. (1989). PCR automation. **In** *PCR technology: Principles and Applications for DNA Amplification*, Ed. Erlich, H. A., Chap. 3, p23-30. Stockton Press, New York.

Ovenden, J. R., Booth, J. D., and Smolenski, A. J. (1997). Mitochondrial DNA phylogeny of red and green rock lobsters (genus *Jasus*). *Mar. Freshwat. Res.*, **48**(8): 1131-1136.

Ovenden, J. R., Bywater, R., and White, R. W. G. (1993). Mitochondrial DNA

nucleotide sequence variation in Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) from Tasmania, Australia. *Aquaculture,* **114**: 217-227.

Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., and Grabowski, G. (1991). *The Simple Fool's Guide to PCR, Version 2. 0*. Department of Zoology and Kewalo Marine Laboratory, University of Hawaii, Honolulu.

Parker, P. G., Snow, A. A., Schug, M. D., Booton, G. C., and Fuerst, P. A . (1998). What molecules can tell us about populations - choosing and using a molecular marker. *Ecology,* **79**: 361-382.

Partis, L. and Wells, R. J. (1996). Identification of fish species using random amplified polymorphic DNA (RAPD). *Mol. Cel. Prob.,* **10**: 435-441.

Ramm, D. C., and Xiao, Y. (1994). Catch, effort and sustainable yield for Australia's northern trawl fishery. In: Australia's Northern Trawl Fishery. Final Report to the Fisheries Research and Development Corporation on Project No. 86/049, 'Assessment of the status, composition and market potential of demersal trawl fish resources in northern Australian waters'. (Ed. D. C. Ramm.) pp.22-35. Northern Territory Department of Primary Industry and Fishery Report No. 32, Darwin

Randall, J. E., Allen, G. R., Steene, R. C. (1990). *Fishes of the Great Barrier Reef and Coral Sea*. Crawford House Press, Bathurst, NSW, Australia.

Richardson, B. J. (1982). Geographical distribution of electrophoretically detected protein variation in Australian commercial fishes II. Jackass morwong, *Cheilodactylus macropterus* Bloch and Schneider. *Aust. J. Mar. Freshwat. Res.,* **33**: 927-931.

Richardson, B. J., Baverstock, P. R., and Adams, M. (1986). *'Allozyme Electrophoresis: A Handbook for Animal Systematics and Population Studies.'* Academic Press: New York, London.

Ritchie PA. Lavoue S. Lecointre G. (1997). Molecular phylogenetics and the evolution of antarctic notothenioid fishes. *Comp. Biochem. Phys.,* **118**: 1009-1025.

Roff, D. A., and Bentzen, P. (1990). The statistical analysis of mitochondrial DNA polymorphisms: C2 and the problem of small samples. *Mol. Biol. Evol.,* **6**: 539-45.

Rothe, G. M. (1994). *Electrophoresis of Enzymes: Laboratory Methods*. Springer

Verlag, Berlin.

Russel, B. C. (1987). Clarification of the use of the family name Synodontidae in the Myctophiformes and Siluriformes. *Copeia,* **1987**: 513-515.

Ryman, N., Lagercrantz, U., Anderson, L., Chakraborty, R., and Rosenberg, R. (1984). Lack of correspondence between genetic and morphological variability patterns in Atlantic herring (*Clupea harengus*). *J. Hered.,* **53**: 687-704.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* **239**: 487-90.

Sainsbury, K. J., Kailola, P. J., and Leyland, G. G. (1985). *Continental Shelf: Fishes of Northern and North-Western Australia.* Clouston & Hall and Peter Pownall Fisheries Information Service, Canberra.

Salini, J. P., Blaber, S. J. M., and Brewer, D. T. (1994). Diets of trawled predatory fish of the Gulf of Carpentaria, Australia, with particular reference to predation on prawns. *Aust. J. Mar. Freshwat. Res.,* **45**: 397-4 11.

Sanger, F., Nicklen,S., Coulson, A.R. (1977). DNA Sequencing with chain- terminating inhibitors. *Proc. Natl. acad. Sci. U. S. A.*, **74**: 5463-5467.

Schmidt, T. R., Bielawski, J. P., and Gold, J. R. (1998). Molecular phylogenetics and evolution of the cytochrome B gene in the cyprinid Genus *Lythrurus* (Actinopterygii, Cypriniformes). *Copeia,* **1998**: 14-22.

Scott, M. P., Haymes, K. M., and Williams, S. M. (1993). Parentage analysis using RAPD PCR. *Nuc. Ac. Res.,* **20**: 5493.

Seeb, L. W. and Kendall, A. W. (1991). Allozyme polymorphisms permit the identification of larval and juvenile rockfishes of the genus *Sebastes. Env. Biol. Fishes,* **30**: 191-201.

Seeb, L. W., and Gunderson, D. R. (1988). Genetic variation and population structure of Pacific Ocean perch (*Sebastes alutus*). *Can. J. Fish. Aquat. Sci.*, **45**: 78-88.

Serventi, M., Harrison, I. J., Torricelli, P., and Gandolfi, G. (1996). The use of pigmentation and morphological characters to identify Italian mullet fry. *J. Fish Biol.,* **49**: 1163-1173.

Shaklee, J. B., Allendorf, F. W., Morizot, D. C., and Whitt, G. S. (1990). Gene

nomenclature for protein-coding loci in fish. *Trans. Am. Fish. Soc.,* **119**: 2-15.

Shaklee, J. B., Salini, J., and Garrett, R. N. (1993). Electrophoretic characterization of multiple genetic stocks of barramundi perch in Queensland, Australia. *Trans. Am. Fish. Soc.,* **122**: 685-701.

Shaklee, J. B., Tamaru, C. S., and Waples, R. S. (1982). Speciation and evolution of marine fishes studied by the electrophoretic analysis of proteins. *Pac. Sci.*, **36**: 141-158.

Shindo, S., and Yamada, U. (1972). Description of three new species of the lizardfish genus *Saurida*, with a key to its Indo-Pacific species. *Uo,* **11**: 1-13, **12**: 1-14.

Slatkin, M. (1994). Gene flow and population strucutre **In** *Ecological Genetics,* Ed. L. A. Real, pp. 3-17, Princeton University Press, New Jersey.

Smith, P. J. and Crossland, J. (1977). Identification of larvae of snapper *Chrysophrys auratus* Forster by electrophoretic separation of tissue enzymes. *NZ. J. Mar. Freshwat. Res.,* **11**: 795-8.

Smith, P. J., and Fujio, Y. (1982) Genetic variation in marine teleosts : high variability in habitat specialists and low variability in habitat generalists. *Mar. Biol.*, **69**: 7-20.

Smith, P. J., Mattlin, R. H., Roeleveld, M. A., and Okutani, T. (1987). Arrow squids of the genus *Nototodarus* in New Zealand waters: systematics, biology, and fisheries. *NZ. J. Mar. Freshwat. Res.,* **21**: 315-326.

Smith, P. J., and Robertson, D. A. (1981). Genetic evidence for two species of sprat (*Sprattus*) in New Zealand waters. *Mar. Biol.,* **62**: 227-233.

Smith, P. J., Wood., and Benson, P. G. (1979). Electrophoretic and meristic separation of blue maomao and sweep. *NZ. J. Mar. Freshwat. Res.,* **13**: 549-551.

Smolenski, A. J., Ovenden, J. R., White, R. W. G. (1993). Evidence of stock separation in Southern Hemisphere orange roughy (*Hoplostethus atlanticus*, Trachichthyidae) from restriction-enzyme analysis of mitochondrial DNA. *Mar. Biol.,* **116**: 219-230.

Smolenski, A. J., Ovenden, J. R., White, R. W. G. (1994). Preliminary investigation of mitochondrial DNA variation in jack mackerel (*Trachurus declivis*, Carangidae) from south-eastern Australian waters. *Aust. J. Mar. Freshwat. Res.,* **45**(4): 495-505.

Solé-Cava, A. M., Vooren, C. M., and Levy, J. A. (1983). Isozymic differentiation of

two sibling species of *Squatina* (Chondrichthyes) in South Brazil. *Comp. Bioch. Phys.*, **75B**: 355-358.

Stepien, C. A. (1992). Evolution and biogeography of the Clinidae (Teleostei: Blennioidae). *Copeia,* **1992**: 375-392.

Sweatman, H. (1984). A field study of the predatory behavior and feeding rate of a piscivorous coral reef fish, the lizardfish *Synodus englemani*. *Copeia*: **1984**:187-194.

Swofford, D. L. (1993). '*PAUP: Phylogenetic Analysis Using Parsimony, ver. 3. 1.*' Laboratory of Molecular Systematics, Smithsonian Institution.

Swofford, D. L., and Olsen, G. J. (1990). Phylogeny reconstruction **In** *Molecular systematics* Ed. D. M. Hillis, and C. Moritz, p.411-501, Sinauer Associates, Inc., Sunderland, Massachusetts.

Swofford, D. L., and Selander, R. B. (1989). '*BIOSYS-1: A Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics.*' Release 1.7. (Illinois Natural History Survey: Champaign.)

Takahashi, K., Terai, Y., Nishida, M., and Okada, N. (1998). A novel family of short interspersed repetitive elements (SINES) from cichlids - the patterns of insertion of SINES at orthologous loci support the proposed monophyly of four major groups of cichlid fishes in Lake Tanganyika. *Mol. Biol. Evol.,* **15**: 391-407.

Taniguchi, N. (1969). Comparative electropherograms of muscle proteins of three species of lizard-fishes referable to the genus *Saurida. Bull. Japan. Soc. Sci. Fish.*, **44**(12);1351-1355.

Testolin, R., and Ferguson, A. R. (1997). Isozyme polymorphism in the genus *Actinidia* and the origin of the kiwifruit genome. *Systematic Botany,* **22**(4): 685-700.

Thresher, R. E. (1984). *Reproduction in Reef Fishes.* T. F. H. Publications, Inc., Ltd. USA.

Thresher, R. E., Sainsbury, K. J., Gunn, J. S., and Withlaw, A. W. (1986). Life history strategies and recent changes in population structure in the lizardfish genus, *Saurida*, on the Australian Northwest Shelf. *Copeia,* **1986**: 876-885.

Torgersen, T., Jones, M. R., Stephens, A. W., Searle, D. E., and Ullman, W. J. (1985). Late quaternary hydrological changes in the Gulf of Carpentaria. *Nature,* **313**: 785-787.

Urho, L. (1996). Identification of perch (*Perca fluviatilis*), pikeperch (*Stizostedion lucioperca*) and ruffe (*Gymnocephalus cernuus*) larvae. *An. Zool. Fen.,* **33**: 659-667.

Utter, F. M. (1994). Perspectives of molecular genetics and fisheries into the 21st century. *Rev. Fish Biol. Fish.,* **4**: 374-378.

Utter, F. M., Milner, G., Stahl, G., and Teel, D. (1989). Genetic population structure of chinook salmon in the Pacific Northwest. *Fish. Bull. U. S.*, **87**: 239-64.

Vawter, L., and Brown, W. M. (1986). Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science,* **234:** 194-46.

Vogler, D. R., and Bruns, T. D. (1998). Phylogenetic relationships among the pine stem rust fungi (*Cronartium* and *Peridermium* spp.). *Mycologia*, **90**: 244-257.

Waples, R. S. (1981). A biochemical and morphological review of the lizardfish genus *Saurida* in Hawaii, with the description of a new species. *Pac. Sci.,* **35**: 217-235.

Ward, R. D., and Grewe, P. M. (1994). Appraisal of molecular genetic techniques in fisheries. *Rev. Fish Biol. Fish.,* **4**: 300-325.

Ward, R. D., Elliott, N. G., Grewe, P. M., and Smolenski, A. J. (1994). Allozyme and mitochondrial dna variation in yellowfin tuna (*Thunnus albacares*) from the Pacific Ocean. *Mar. Biol.,* **118**: 531-539.

Waters, J. M., White, R. W. G. (1997). Molecular phylogeny and biogeography of the Tasmanian and New Zealand mudfishes (Salmoniformes, Galaxiidae). *Aust. J. Zool.,* **45**: 39-48.

Waters, J. M., White, R. W. G. (1997). Molecular phylogeny and biogeography of the Tasmanian and New Zealand mudfishes (Salmoniformes , Galaxiidae). (vol 45, pp 39, 1997). [Correction, Addition] *Aust. J. Zool.,* **45**: 671.

Whitmore, D. H. (1990). Isoelectric focusing of proteins. **in** *Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management*, Ed. D. H. Whitmore, pp. 81-105. CRC Press, Boca Raton.

Wilson, A. C., Cann, R. L., and Carr, S. M. *et. al.* (1985). Mitochondrial DNA and two perspectives on evolutionary genetics. *Biol. J. Lin. Soc.,* **26**: 375-400.

Wolanski, E., Ridd, P., and Inoue, M. (1988). Currents through Torres Strait. *J. Phys. Ocean.,* **18**: 1535-45.

Wright, S. (1931). Evolution in Mendellian populations. *Genetics*, **16**: 97-159.

Wright, S. (1943). Isolation by distance. *Genetics,* **28**: 114-38.

Wright, C A., Richards, C. M., Frost, J. S., Camozzi, A. M., and Kunz, B. J. (1983). Genetic mapping in amphibians. *In Isozymes: Current Topics in biological and Medical Research, Vol. &. Molecular Structure and Regualtion*, pp 287-377, Liss, New York.

Wright, J. M., and Bentzen, P. (1994). Microsatellites: genetic markers for the future. *Rev. Fish Biol. Fish.,* **4**: 384-388.

Yamada, U. and Ikemoto, R. (1979). A quick identification of three species of lizardfish, *Saurida,* in Japanese and adjacent waters. *Bull. Seikai Reg. Fish. Res. Lab.,* **52**: 61-69.

Yamaoka, K., Nishiyama, M., and Taniguchi, N. (1989). Genetic divergence in lizardfishes of the genus *Saurida* from Southern Japan. *Jap. J. Icht.,* **36**: 208-219.

Yang, H. (1997). Ancient DNA from pleistocene fossils - preservation, recovery, and utility of ancient genetic information for quaternary research. *Quat. Sci. Rev.,* **16**(10): 1145-1161.

Yano, K., and Musick, J. A. (1992). Comparison of morphometrics of Atlantic and Pacific specimens of the false catshark, *Pseudotriakis microdon*, with notes on stomach contents. *Copeia,* **1992**: 877-886.

Yeatman, J., and Benzie, J. A. H. (1994). Genetic structure and distribution of *Photololigo* spp in Australia. *Mar. Biol.,* **118**: 79-87.